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Physiological and Genetic Studies of Deoxynojirimycin
Production in *Streptomyces* Species.

by

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A thesis presented for the degree of
Doctor of Philosophy.

Department of Biological Science
University of Warwick.

September 1993

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ACKNOWLEDGEMENTS

Primarily, I wish to thank my supervisor Dr. Elizabeth Wellington for her help and advice throughout the project and the proof-reading of the thesis on completion. The neverending source of ideas and suggestions and her grasp of the subject material made me feel my thinking was quite linear in comparison.

Concerning the technicalities of *Streptomyces* cloning, there is nobody better than Dr. David Hodgson whose understanding of molecular biology is truly outstanding. I also wish to thank Dr. Derek Hood whose constant patience never ceased to amaze me, despite my apparent floundering during the compilation of the gene libraries. I also wish to thank Dr. David McDowell, for having the time to explain to me many of the aspects of molecular biology.

The work involving the use of enzyme kinetics reflects input from Dr. Ken Flint, his help with the statistical treatment of screening results was also appreciated. In the laboratory I thank Mrs Jane Green the Chief Technician for her helpfulness and efficiency. Dr Paul Mullins was a great help thoroughly proof-reading the thesis and providing sound advise on matters external to biology.

I extend my gratitude to Dr Neil Porter of Xenova, for his advice on how to analyse screening data in the most logical fashion. The use of many of the facilities at Xenova was central to the project, particularly the

finance office for paying my expense account, "Yes I really was there all those days".

Finally, I thank friends and colleagues at the University and Xenova for an enjoyable 3 years (and a few months) doing this PhD.

Declaration

I declare that this thesis has been compiled by myself from the results of research conducted under the supervision of Dr. E.M.H. Wellington and Dr. N. Porter and has not been used in any previous application for a degree. All the sources of information have been acknowledged by means of a reference.

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Abbreviations

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
SCP2	<i>Streptomyces coelicolor</i> plasmid.
SLP2	<i>Streptomyces lividans</i> plasmid.
CCC	Closed covalently circular
DNJ	Deoxynojirimycin
NOJ	Nojirimycin
<i>ermE</i>	Erythromycin resistance gene
HIV	Human immunodeficiency virus
gp	Glycoprotein
DMJ	Deoxymannojoirimycin
MOJ	Mannojoirimycin
ISP4	Inorganic salts and starch agar
ISP5	Starch and glycerol agar
MGA	Minimal glucose agar
TE	Tris-EDTA buffer
kb	Kilobase pairs
TK24	<i>Streptomyces lividans</i> resistant to streptomycin
YEME	Yeast extract malt extract
TSB	Tryptone soya broth
Hg	Mercury
SDS	Sodium dodecyl sulphate
ZX1	<i>Streptomyces lividans</i> modification mutant
a.u./min	Absorbance units per minute
K _i	Enzyme inhibition constant
K _m	Substrate concentration that gives half V _{max}
V _{max}	Maximal rate of enzyme substrate reaction
GOD-PAP	Glucose oxidase peroxidase kit
S.	Denotes <i>Streptomyces</i> species

ABSTRACT

Genomic libraries of S.lavendulae and S.subbrutilus were constructed in S.lividans using the technique of shotgun cloning. S.lividans is a genetically well characterised recipient for heterologous DNA, but plasmid deletions or the entry of plasmids with small inserts occurred during transformation of the DNA into S.lividans. This was not due to a straightforward restriction-modification system as this possibility was checked using the KC301 phage. Several gene libraries were produced, using high and low copy number plasmids, and the resultant transformants screened.

The nature of deoxynojirimycin (DNJ) and its lack of microbial activity prevented use of a bioassay. However, the inhibition of α -glucosidase by DNJ was exploited by development of a quantitative assay system for DNJ and nojirimycin (NOJ). The assay could not only detect DNJ and NOJ-producing clones, but could also assess the titre of DNJ and NOJ in culture broths. The assay was used to demonstrate differential production of DNJ and NOJ by selected Streptomyces strains of cluster 61 (the S.lavendulae species group). The assay also confirmed the effectiveness of using microtitre plates as an effective screening procedure. The microtitre screening programme generated further data and statistical treatment of the results delimited the number of isolates for further examination. No DNJ-producing colony was detected and examination of the size of the DNA inserts showed almost all were too small to contain the DNJ gene cluster.

Additionally, blocked DNJ production mutants were characterised by the feeding of NOJ, one of the mutants successfully converted NOJ to DNJ.

CHAPTER ONE

INTRODUCTION

Introduction

Actinomycetes are Gram-positive bacteria with a DNA composition abundant in guanine and cytosine (69-78%). A range of chemical techniques are available to determine lipid composition, wall sugar and amino acid composition of bacteria. These traditional taxonomic methods have produced taxonomic groups which were heterogeneous (Lechevalier and Lechevalier, 1970). The groups of actinomycetes in Bergey's Manual Of Systematic Bacteriology Vol. 4 (1989) are mainly grouped by a selection of chemical and morphological techniques, together with sequence data from 16S ribosomal RNA.

More recently molecular methods involving hybridisation, sequencing of nucleic acids and especially comparison of 16S ribosomal RNA sequences have established new groups of relatedness for actinomycetes and the prokaryotes in general (Fox and Stackebrandt, 1987). The first dendrograms summarising prokaryotic phylogeny based on 16S ribosomal RNA oligonucleotide catalogues were published in 1980 (Fox et al., 1980). However, a complete and comprehensive phylogeny of the actinomycetes based on 16S RNA sequences is not yet available. The procedure is also better for some groups than others. For example, good relatedness was found

between sequencing data and ribosomal RNA cistron similarity for the actinoplanetes, maduromycetes, nocardiform actinomycetes and streptomycetes (Stackebrandt and Woese 1981). Inter- and intra-generic relationships of *Actinomyces* have been determined by comparing the 16S rRNA sequences produced by reverse transcriptase. Stackebrandt and Charfreitag (1989) showed that *Actinomyces bovis*, *A. visosus*, *A. naeslundii*, *A. odontolyticus* and *A. israelii* constituted a genetically well defined species and formed a phylogenetically coherent cluster. The 16S sequencing data showed the *Actinomadura* and the *Dermatophilaceae* contained very diverse taxa. Additionally, the *Micromonospora* were shown to be more closely related to the sporangia-forming actinomycetes, like the *Actinoplanes* and the *Dactylosporangium*, rather than other monosporic genera, such as the *Thermomonospora* or the *Thermoactinomyces*.

1.2 *Streptomyces* taxonomy.

The vegetative hyphae of *Streptomyces* are 0.5-2 μ m in diameter and produce an extensively branched mycelium. When the aerial hyphae are mature three or more spores are produced and some species even have spore chains on their substrate mycelium. The mycelium always forms a discrete colony and sporangia or motile spores are never present. The cell wall contains diaminopimelic acid (LL-DAP) but never contains the sugars arabinose, galactose or xylose. The fatty acid constitution consists of saturated straight chain and iso- and anteiso- branched

fatty acids with unsaturated fatty acids absent. *Streptomyces* are members of the larger streptomycete group which have three characteristic features. Streptomycetes have a vegetative mycelium, aerial mycelium bearing chains of arthrospores (sporophores) and the arthrospores themselves. Controversy existed over whether the genera *Actinopycnidium*, *Actinosporangium*, *Chainia*, *Elytrosporangium*, *Kitasotoa* and *Microellobosporia* were separate groups. They could be distinguished from *Streptomyces* using morphological criteria and yet Goodfellow et al. (1986abcd) maintained they were synonyms because they had many phenetic, chemical, molecular and genetic characters in common. A similar problem existed between the *Streptomyces* and the *Streptoverticillium* which are differentiated from *Streptomyces* by the presence of verticilliate sporophores during sporulation. Phenetic data from Williams et al. (1983) supports the continued recognition of *Streptoverticillium* as a distinct group, whilst work by Witt and Stackebrandt (1990) using 16S ribosomal RNA proved they were very closely related (Fig 1.1).

The first large scale screening of actinomycetes for bioactivity led to the discovery of Streptomycin (Schatze et al., 1944). Species of *Streptomyces* produce a wide variety of pigments that colour the vegetative and aerial mycelia, and many isolates were discovered that could not be assigned to any taxon.

.

Fig 1.1

Phylogenetic Tree Showing the Intrafamily Relationships of Streptomycetaceae based on comparison of 520 nucleotides from the 16S ribosomal RNA.

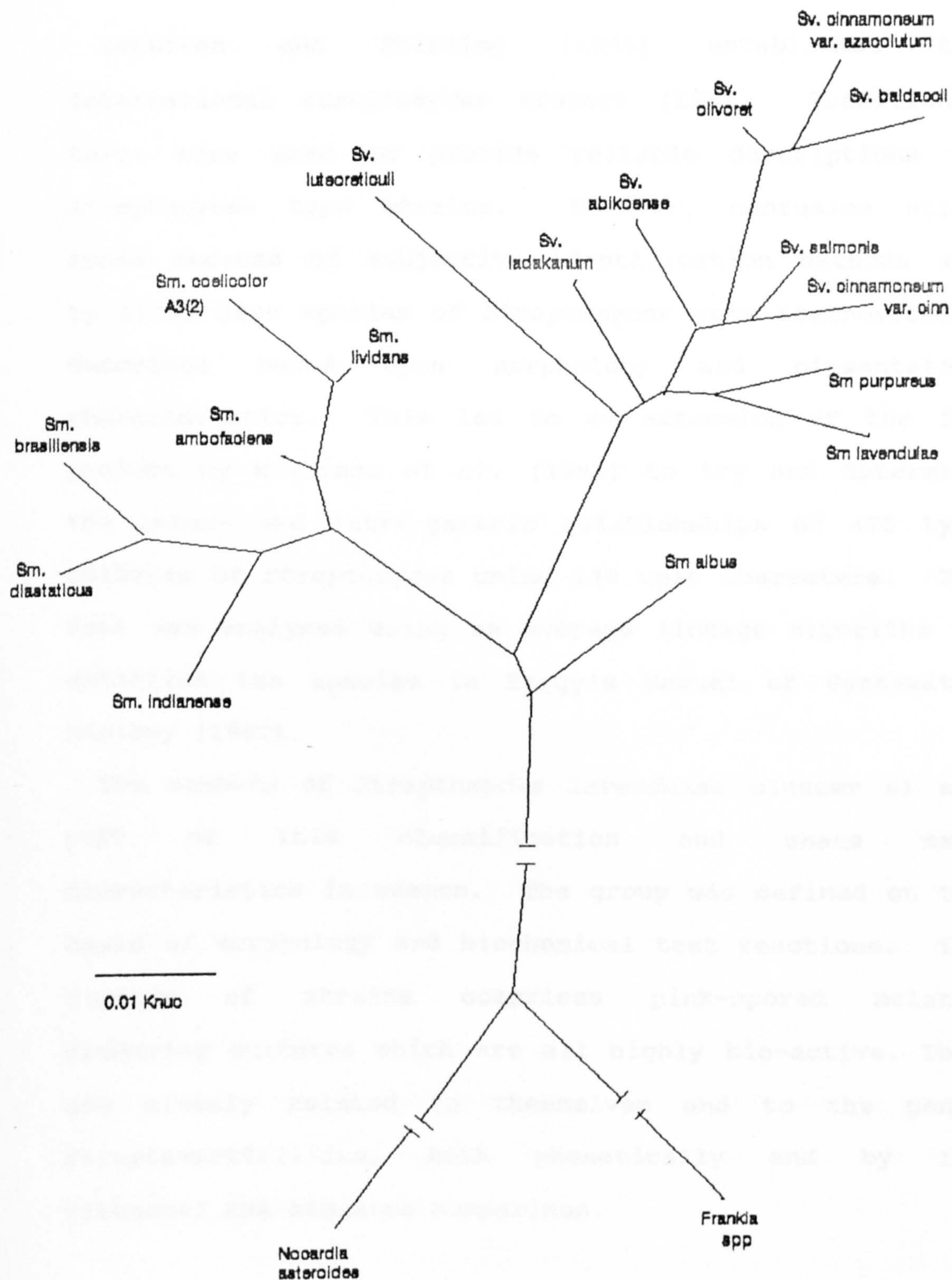
The root was determined using Nocardia asteroides and a Frankia strain as reference organisms. The bar corresponds to 0.01 Knuc. The gaps in the branches to the reference organism span 0.05 Knuc.

Sv - Streptoverticillium

Sm - Streptomyces

Knuc - Thousand nucleotides

Redrawn from Witt and Stackebrandt: 1990.



Gottlieb and Shirling (1966) established the International *Streptomyces* Project (ISP). Traditional tests were used to provide reliable descriptions of *Streptomyces* type strains. However, confusion still arose because of subjective identification methods and by 1970, 3000 species of *Streptomyces* were taxonomically described based upon morphology and pigmentation characteristics. This led to an extension of the ISP project by Williams et al. (1983) to try and determine the inter- and intra-generic relationships of 475 type cultures of *Streptomyces* using 139 unit characters. The data was analysed using an average linkage algorithm to establish the species in Bergy's Manual of Systematic Biology (1989).

The members of *Streptomyces lavendulae* cluster 61 are part of this classification and share many characteristics in common. The group was defined on the basis of morphology and biochemical test reactions. The cluster of strains comprises pink-spored melanin producing cultures which are all highly bio-active. They are closely related to themselves and to the genus *Streptoverticillium*, both phenetically and by 16S ribosomal RNA sequence comparison.

1.3 Ecology and life cycle.

In soil, organic particles are colonised by substrate mycelia. Hyphae then grow around the soil particles, and chains of arthrospores are produced. Almost all of the *Streptomyces* genera produce aerial mycelium, which at maturity forms chains of 3 to many spores. Only a few species, for example *S. carpinensis*, bear spores on the substrate mycelium. The spores, whether of substrate or aerial origin, are always non-motile. If nutrients are limiting the germ tube itself may be transformed into sporogenous hyphae and then converted to chlamydospores. The sequence of events was originally documented by Pfennig (1958). The generation time for *Streptomyces* species was determined gravimetrically in batch culture to be 1.14-1.88 hours (Flowers and Williams, 1977). However, the generation time in woodland soil for a *Streptomyces* population was estimated to be 1.7 days by Mayfield et al., (1972). Most of the population existed as spores and there were significant increases in the viable counts for *Streptomyces*, when starch, chitin, casamino acids or fungal mycelium were added to the soil (Williams, 1978).

Streptomyces are widely distributed in both terrestrial and aquatic habitats. However, their presence in freshwater and marine environments is mainly due to being washed in from the soil. The *Streptomyces* are quite capable of survival in lake sediments but do not usually

belong to the aquatic ecosystem (Korn-wendish and Kutzner, 1992). Most are strict saprophytes, however, some have parasitic associations with plants (*S.scabies* and potatoes) or animals (*S.somaliensis*). Evidence for their widespread occurrence was well documented by Goodfellow and Williams (1986). Soil, fodder and compost were highlighted as primary reservoirs for a wide variety of thermophilic and mesophilic *Streptomyces* species and their spores. Lacey (1978) examined the ecology of actinomycetes in fodders and related substances and found water content to be critical. At 25-35% moisture, the temperature increases to between 45-55°C and mesophilic *Streptomyces* predominate. At higher moisture content temperatures of 70°C were attained and *Streptomyces* were replaced by thermophilic actinomycetes. Organic substrates from leaf litter, or root tips stimulate germination, growth and colonisation with localised production of spores when nutrients are exhausted (Mayfield et al., 1972). The survival of the *Streptomyces* species was by spores since they have a thicker coat and are more hydrophobic, features lending protection from adverse environmental conditions (Sharples et al., 1974). The spores are not as resistant as bacterial endospores but have a relatively low endogenous metabolism and were more resistant to heat and desiccation than hyphae (Ensign, 1978). They are also resistant to frost, hydrostatic pressure and anaerobic conditions as a consequence of water saturation. *Streptomyces* hyphae are strictly aerobic but can survive

at low oxygen tensions. In dry soils absolute numbers of *Streptomyces* decline but relative to other bacteria their number may proportionately increase (Locci, 1976).

The primary ecological function of *Streptomyces* is the degradation of recalcitrant polysaccharides, starch, pectin and chitin. Mordarski et al. (1970) showed that amylases as extracellular complex enzymes were widespread in the actinomycetes. Even cellulose can be degraded by *Streptomyces* and several endoglucanases and exoglucanases have been found in *Streptomyces* species. Especially within the thermophilic species which seem more significant in cellulose degradation.

Streptomyces can also degrade lignin usually bound up as lignocellulose in grass and wood (Crawford and Sutherland, 1979). The lignin component was once thought only degradable by fungi; but this has now been refuted. However, the mechanism for lignin degradation at the enzyme level appears different. Four enzymes are involved; one of which, the enzyme cleaving the β -ether linkages, remains unisolated (Crawford, 1988). Xylan is also part of the lignocellulose complex and was broken down by the lignolytic *Streptomyces* (Deobald and Crawford, 1987).

Streptomyces are chemo-organotrophs, they are not fastidious and need only a suitable carbon source, nitrogen source and mineral salts. They have no requirements for vitamins or growth factors. Carbon sources vary but widely used are: cellobiose, glycerol, D-mannose and trehalose. The nitrogen sources most

commonly used are: ammonium, L-arginine, L-asparagine and nitrate. Spore production by *Streptomyces* is usually the most abundant when media of a high carbon to nitrogen ratio is used (Korn-Wendisch and Kutzner, 1992).

Distribution in the natural environment is constrained by temperature and pH. Most *Streptomyces* are mesophiles with growth confined to temperatures between 10°C and 37°C. The optimal temperature for growth of mesophiles is between 25°C and 35°C. Usually these *Streptomyces* are thermotolerant but some, for example *S.thermoflavus* are truly thermophilic and do not grow at temperatures below 37°C.

The majority of *Streptomyces* are neutrophiles in culture with optimal growth between pH 5 and pH 9. Only a few of these organisms grow at pH 4.3. Many acidoduric and acidophilic strains have been isolated from acidic soils (Williams and Mayfield, 1971). The many neutrophiles found in the acidic environment are accounted for by the resistance of their spores and the non-uniform acidity of the environment (Williams et al., 1971). The biochemistry of the acidophiles is modified so they produce diastases and their chitinases have optimal pH below equivalent enzymes of neutrophiles (Williams and Robinson, 1981). Pharmaceutical companies have also exploited this habitat in the search for novel isolates (Langley et al., 1991). Isolates from extreme environments have been found to contain a larger proportion of producers of novel compounds.

1.4 Antibiotic production and antibiosis

It was naturally assumed that antibiotics were produced in soil by *Streptomyces* species due to the abundant laboratory evidence of antibiotic production. However, direct evidence of production in soil was minimal. One of the best examples monitored populations of *Salmonella dusseldorf*, *Streptomyces lividans* and *Streptomyces bikiniensis* (Turpin et al., 1992). In non-sterile amended soil *Streptomyces lividans* and *Streptomyces bikiniensis* both reduced the survival of *Salmonella dusseldorf*. The most dramatic reduction was caused by *Streptomyces bikiniensis* providing evidence of antibiosis caused by the production of streptomycin by *Streptomyces bikiniensis*. The difficulty of extracting antibiotics from soil probably reflects the instability of the antibiotic in soil, the insensitive detection methods available and the adsorption of the antibiotic to soil colloids, especially clay.

Despite these problems the effect of antibiosis has been studied for its impact on plant pathogens. This of course was indirect evidence of antibiotic production in soil. Wellington and Al-Jawadi (1985) obtained some control of a fungal root infection by polyene production from *S.lavendulae* and *S.polychromogenes* when spores of these *Streptomyces* species were used as a root dip. Soil-borne diseases have also been controlled by amending soils with chitin and fungal cell wall material. Kundu

and Nandi (1985) found that damping off caused by *Rhizoctonia* could be controlled by addition to the soil of cellulosic waste products. In a similar study (Ebben and Spencer, 1978) inoculated growth substrates of cucumbers with *S.griseus* cells which produce an antibiotic active against *Phomopsis sclerotioides*. There was some reduced incidence of the disease in straw grown plants, but not when grown on peat. Sneh and Henis (1972) argued that it was not the antifungal antibiotics produced by *Streptomyces* which were important but merely the capacity to instigate lytic effects on certain fungi.

Antibiosis has also stimulated an investigation into the evolution and function of these secondary metabolites. Stone and Williams (1992) concur with the view that antibiotics are produced in an antagonistic capacity. This is because the pathways of secondary metabolites are sophisticated and are energetically expensive. The natural products produced usually inhibit microbes and the producers themselves usually lack immune systems. Secondary metabolites are not artifacts of growth-culture conditions because resistance genes have evolved in close association with the biosynthetic genes. Hopwood (1988) identified the genes for three antibiotics in *S.coelicolor* and all are clustered with their respective resistance genes. Stone and Williams (1992) argue that if *Streptomyces* species never produced antibiotics in nature why would it be necessary to have a functional resistance gene always in close association with the biosynthetic genes?

1.5 The general physiology of *Streptomyces*.

1.5.1 The central pathways of metabolism involving carbon.

There was good evidence for the existence of an Emden-Meyerhof-Parnas and hexosemonophosphate pathway in *Streptomyces coelicolor*, *S. reticuli* and *S. scabies*, in addition to a tricarboxylic acid cycle (TCA), which was complete and active (Cochrane, 1961). Hostalek et al. (1969) reported that all the usual TCA enzymes were present and correlated these enzymes in some strains with the production of chlorotetracycline.

All bacteria except *Streptomyces*, that possess the Emden-Meyerhof-Parnas or the hexosemonophosphate pathway can grow anerobically on glucose or related sugars. *Streptomyces* however, are obligate aerobes, although Hockenhull et al. (1954) reported the production of lactate by *S. griseus* under low oxygen tensions. In addition to a TCA cycle *Streptomyces* do have a glycolysis pathway with pyruvate being the end product.

Streptomyces can utilise a wide variety of carbon sources but only one thermophilic strain has been shown to be a true autotroph (Bell et al., 1987). The strain can grow on carbon monoxide (CO) and contains an enzyme from the Calvin carbon dioxide fixation cycle which acts as a CO oxygen reductase.

1.5.2 Carbon storage compounds

The two main storage compounds in *Streptomyces* species are trehalose and glycogen. Furthermore trehalose has been found in the vegetative mycelium of every *Streptomyces* examined to date. Hey-Ferguson *et al.* (1973) investigated the two enzymes involved in trehalose anabolism, trehalose phosphate synthase and trehalose phosphate phosphatase and the enzyme of trehalose catabolism trehalase. During germination of spores in a nutrient medium trehalase activity increased and trehalose fell, the level of trehalose phosphate phosphatase also fell until outgrowth had begun. These observations were in accord with trehalose being the main carbon and energy source for *Streptomyces* spores (McBride and Ensign, 1987). Trehalose also has a secondary function as an osmoprotectant. During catabolism of trehalose there was a concomitant loss of dehydration resistance. Addition of exogenous trehalose led to a restoration of the dehydration resistance.

Glycogen was initially reported within *Streptomyces* spores by Hey-Ferguson *et al.* (1973), but a more accurate monitoring of the accumulation and disappearance of the polysaccharide granules in *S.viridochromogenes* was done by Brana *et al.* (1980). The granules were characterised as glycogen and disappearance of the granules coincided with production of sporulation septa in aerial hyphae (Brana *et al.*, 1982). When the sporulation septa were

complete the cells filled with granules but these disappeared for the second time once full maturation had been achieved. Glycogen is a temporary carbon store in an insoluble but mobile form.

1.5.3 Central pathways of metabolism involving nitrogen.

1.5.4 Cyclic AMP (cAMP)

Many catabolic enzymes are dependent on cAMP binding, for control of their regulation. The presence of cAMP-CRP is essential for the expression of genes, even if the inducer of the operon is present. The cAMP-CRP binds to a particular sequence of the DNA which, (in the absence of repressors) initiates the binding of RNA polymerase. The concentration of cAMP is increased by synthesis of adenyl cyclase. The cAMP is decreased by production of phosphodiesterase, or simply exported out of the cell.

1.5.5 Glutamine synthases

Glutamine synthase (GS) is regulated by ammonia in enteric bacteria. For example ammonia inactivates the GS of enteric bacteria by adenylation. GS in *Streptomyces* was initially discovered by Tronick et al. (1973) in *S.rutgersensis* and *S.diastatochromogenes*, but in *Streptomyces* the enzyme was not inactivated by adenylation. This work was developed by Wax et al. (1982) when the GS system of *S.cattleia* was inactivated

by ammonia shock and adenylation. Additional work involving immunological cross reactivity and protein sequencing indicated the GS of *Streptomyces* was similar to the GS of enteric bacteria. This conclusion was confirmed when Xia and Jiao (1986) isolated an identical enzyme, of 12 identical subunits in two hexagonal rings, from *Streptomyces* and the enteric bacteria. Recently, interest has focused on the existence of two forms of GS, one of which can be inhibited by bialophos. Behrmann et al. (1990) isolated a gene from *S.viridochromogenes* which was homologous to the *gln II*, GS enzyme of eukaryotes and *Rhizobium* spp. This gene was supplementary to the usual GS (*glnA*) gene and when other *Streptomyces* were probed all were found to have both genes.

Glutamate synthase (GOGAT) has been found in all *Streptomyces* so far examined. However, there appears to be no co-ordinated regulation between GOGAT, GS or glutamate dehydrogenase (GDH). In *S.coelicolor* GOGAT is dependent on the nitrogen source and the concentration of GOGAT was seven times lower grown on alanine than a complex nitrogen source (Fisher, 1989). Similarly, in *S.venezuelae* NADH-dependent GOGAT is regulated by the concentration of nitrogen. High ammonia concentrations in the medium stimulated GOGAT and GDH, whilst, activity was repressed in poor nitrogen sources (Shapiro and Vining, 1983). The reverse was true with *S.clavuligerus* and *S.noursei*, GS was regulated but GOGAT was not. However, in *S.coelicolor* and *S.hygroscopicus* both enzymes were regulated by the nitrogen source.

Streptomyces, in common with most bacteria obtain nitrogen from glutamine and glutamate, although there is however no evidence that *Streptomyces* can fix nitrogen. *Streptomyces* do contain nitrate reductase and in a survey of *Streptomyces* by Mansour and Shady (1984) there was variation in nitrogen reduction capacity due to medium components. Production of nitrate reductase was highest in *S.fulvoviridis*, when glucose was used as a carbon source, but completely repressed when sorbose or sucrose was used. Shapiro and Vining (1984) showed a diauxic growth of *S.venezuelae* on medium containing ammonium and nitrate. The ammonia was catabolised first before utilization of nitrate, which was preferentially used before proline.

The nitrogen obtained by these methods is used to manufacture proteases, peptidases, nucleotides and amino acids. A more detailed account of proteases and peptidases can be found later in this Chapter.

1.5.6 Nutrition and its effect on antibiotic production

Carbon repression of secondary metabolite production is called catabolite repression and whole operons are repressed as long as the carbon source is present. It is a widely reported phenomenon, for example chloramphenicol production is repressed by glucose and lactose using cultures of *S.venezuelae* (Chatterjee and Vining, 1981). Catabolite repression also occurred during the production of Cephalosporin by *Cephalosporium acremonium*. When

excess glucose was present penicillin N accumulated, while Cephameycin C declined due to repression of deacetoxycephalosporin C synthase, (Zanca and Martin, 1983). Most commercial fermentations of polyenes use slow feeding of glucose as the carbon source, to a concentration of 7-9.5% (w/v) to avoid catabolite repression, retarded growth and abnormal fermentation. This process was initially utilized to improve penicillin fermentation yields and has since been extended to candicidin and candihexin (Martin and McDaniel, 1975).

Vilches et al. (1990) documented a detailed examination of the production of the macrolide oleandomycin by *S. antibioticus*. *S. antibioticus* grew in a variety of carbon and nitrogen media but produced oleandomycin most effectively when the carbon source was consumed slowly. Glucose, glycerol, trehalose and soluble starch all led to high biomass but low oleandomycin titre. Catabolite repression was deemed responsible rather than pH since the pH remained constant during the production phase of the antibiotic. Fructose was the most effective producer but only 20% of the initial quantity was used. Aspartic acid was added in conjunction with the fructose and the aspartic acid was used preferentially as a source of carbon and nitrogen. Only after 60 hours when most of the amino acid had been exhausted was fructose finally utilised as a source of carbon. Aspartic acid and sodium nitrate gave the highest specific antibiotic production although biomass levels were not high.

Catabolite repression is also prevalent in

fermentations using *Streptovercillium*. Soliveri et al. (1988) examined the production of two polyenes PA5 and PA7 and obtained results similar to Martin and McDaniel (1975). D-glucose and glycerol supported the highest growth rates and production of PA5 and PA7 occurred just after the growth maximum. After 60 hours the growth ceased when the glucose level fell to 55mM and 70mM. D-glucose initially stimulated production which was optimal for both antibiotics at a concentration of 40mM. When the glucose was exhausted production ceased.

Further studies on glucose repression were made by Hodgson (1982) using *S.coelicolor*. Arabinose transport was repressed by glucose at the transcription level. There were two glycerol transport systems, one was an active transport system and was glucose repressed. The other operated by facilitative diffusion and was inhibited but not repressed. Active transport systems for galactose and fructose were both inhibited by glucose. The metabolic enzymes for galactose and fructose were inducible but only in the absence of glucose. The repression of whole operons and metabolic enzymes was a previously known feature of glucose repression. However, the influence on the uptake of the sugars was a new mechanism of repression.

Additional observations related to catabolite repression are that strains that produce high titres of antibiotic are less sensitive to repression. Enhanced production was also observed in cultures where the carbon source was derived from more than one amino acid

(Bushall, 1988).

Work has been done to try to determine the presence of a glucose-mediated cyclic AMP regulation system in *Streptomyces*, as there is in enterics. This does not seem to occur in *Streptomyces* since the addition of cAMP to *S.kanamyceticus* relieved the glucose repression of kanamycin. However, the situation was unresolved as there was a non-reversal of glucose repression by cAMP in actinomycin production.

Gersch et al. (1979) examined phosphate inhibition and the role of cyclic AMP in turimycin production. Phosphate inhibits turimycin production completely at 0.1M phosphate. However, a mutant was found that could produce turimycin at concentrations of 5-10mM. The phosphate increased intracellular cyclic AMP content. However a second elevation of cyclic AMP, normally seen in the growth phase was prevented by the phosphate. The cyclic AMP was able to overcome the phosphate repression and a protein band implicated as responsible for turimycin biosynthesis reappeared.

Nitrogen and ammonia were implicated in the regulation of secondary metabolites. Excess ammonia activates glutamate dehydrogenase (GDH) decreasing production of cephalosporin. A similar control system has been observed in *S.venezulae* for the production of chloramphenicol (Chatterjee and Vining, 1982). Shapiro and Vining, (1983) suggested GDH and the GS GOGAT systems are linked to repress secondary metabolism.

The production of cephalosporin by *S.clavuligerus* was

the focus of a study to determine the optimal nitrogen source. When added to a defined medium certain amino acids such as asparagine and glutamate gave the best results, while arginine was also effective. Increasing concentrations of ammonium could shift antibiotic production, to production within the stationary phase, when amino acids were the major nitrogen source (Arhonowitz and Demain, 1977).

Soliveri et al. (1988) examined the production of the polyenes PA5 and PA7 by *Streptoverticillium*. Optimal production was achieved with L-proline (17Mm) and glycine (25Mm) as nitrogen sources. Supplementary additions of L-valine or cysteine (17Mm), inhibited production of the polyenes, but did not inhibit cell growth. Additional inorganic salts or complex nitrogen sources caused no enhancement of production.

An empirical approach is often the only method available to optimise fermentation broths, due to the almost exclusive use of complex media. For instance soya bean meal has frequently been cited as responsible for slowly releasing repressive nutrients in cultures (Booth, 1971). However, slow breakdown of complex media does prevent an accumulation of repressive amino acids and ammonium (Demain, 1986).

The other nutrient cited to have an influence on the regulation of antibiotics is phosphate. Two mechanisms of control were proposed, either directly affecting biosynthetic phosphatases, or determining the level of ATP which indirectly affects regulation. These

mechanisms of control were similar to the methods previously outlined for nitrogen regulation. The ability of phosphates to repress enzymes and inhibit candicidin production was demonstrated by Liras et al. (1977). Candicidin production was found to be initiated by phosphate depletion. When 10mM phosphate was added at the beginning of fermentation, no candicidin was produced because of the repressive effect on the biosynthetic enzymes in the polyene pathway. P-aminobenzoic acid synthase (PABA) was the key enzyme in candicidin production, its decline in activity was paralleled by a decline in candicidin production under phosphate repression.

Repression of biosynthetic enzymes by extra cellular phosphate was reported in tetracycline production, (Bahal et al., 1979), candicidin production (Gil et al., 1981), and with cephamycin and clavulanic acid (Lebrihi et al., 1987). Thienamycin and Cephamycin C production by *S.cattleia* were also phosphate regulated. Thienamycin in chemostat culture was only produced under phosphate limiting conditions. Cephamycin C production occurred at low specific growth rates, regardless of other nutrients present (Lilley et al., 1982).

Phosphate affects antibiotic regulation by altering the intracellular level of ATP, which usually drops immediately prior to secondary metabolite production. Madry et al. (1979) found ATP reduction suppresses the anabolic reactions of primary catabolism. Biosynthesis of candicidin was inhibited in *S.griseus* when

intracellular phosphate was increased (Martin and Demain, 1976).

1.5.7 Aeration and oxygen supply

Aeration which directly affects oxygen tension is influential in determining antibiotic titre. Martin and McDaniel (1975) monitored oxygen levels during the production of candicidin and candihexin. During stationary phase there was a large increase in oxygen uptake. Oxygen tension was at 90% saturation during logarithmic phase, but declined to 50% saturation during the stationary phase. The onset of polyene production occurred at the final stage of the logarithmic phase when only DNA was being synthesised slowly. Polyene production was initiated when oxygen uptake and glucose utilisation were maximal and respiration rate decreased dramatically with production of either polyene. The uptake of oxygen was arrested after 60 hours and production ceased. Cessation of production was not caused by pH change but probably reflected the utilisation of NADPH. The NADPH is used to reduce the highly oxidised polyketide chain to the reduced polyene macrolide. This leaves little NADPH for respiration.

Yegneswaran et al. (1988) monitored oxygen tensions and cephamycin titres in cultures of *S.clavuligerus*. Antibiotic levels were unaffected by reduced oxygen for the initial 50 hours of fermentation. When growth ceased antibiotic concentration dropped three fold under reduced

oxygen, while antibiotic concentration was stable when air was used. Yegneswaran et al. (1988) suggested that the enzymes for hydrolysing antibiotics were regulated by aeration conditions. Higher dry cell weight was observed with less oxygen. It is well documented that several reactions in the cephalosporin biosynthetic pathway have a requirement for oxygen (Ahronowitz and Demain, 1977). Despite this, high oxygen tension inhibited growth. Optimal oxygen concentration for biomass production was different from the level for the optimal production of antibiotic concentration. The level of carbon dioxide was also significant, since high levels inhibit antibiotic formation. Carbon dioxide stripping with increased air flow depresses the hydrolytic enzymes and increased antibiotic titre.

1.6 Genetic manipulation of *Streptomyces*.

Streptomyces contain one circular chromosome and can possess a variety of plasmid types. The genome size is approximately $3.7-5 \times 10^9$ daltons of DNA per haploid genome (10,000kb), which is 1.5 - 2 times as large as *E.coli*. The genome is complex, Antonov et al. (1977) calculated that 5% of the genome of *S.coelicolor* consists of sequences repeated in a few copies per chromosome.

1.6.1 *Streptomyces* plasmids

Many streptomycetes carry detectable extra chromosomal elements and some hosts are able to contain more than one plasmid type. The plasmids range in size from 1.8 - 340kb and a copy number between 0.2 and 600, they may account for up to 20% of the total cellular DNA. The largest is the SCP1 giant linear plasmid (340kb) from *S.coelicolor* which has been found to code for antibiotic biosynthesis genes. *Streptomyces* also contain "mini-circles" which are small closed covalently circular (CCC) molecules present at low copy number. The IS117 mini-circle is 2527bp and is present in *S.coelicolor* at one copy per 10-20 chromosomes.

Constraints on replication and copy number are poorly understood. Hopwood et al. (1986) studied the minimal replicon size using pUC6 and SCP2 and found that some

plasmids less than 2kb were viable replicons. Furthermore deletion of part of the plasmid DNA resulted in an increased copy number. In a similar study by Larson and Hershberger (1986), 1.4kb was found to be the minimum replicon size for SCP2 derivatives.

Conjugation is dependant on plasmid sex factors, which many strains have, although frequencies of recombination for certain strains are low. *S.lividans* shows good recombination frequencies once the SLP2 plasmid is eliminated (Hopwood et al., 1983)

It is possible to determine the position of genes on an *E.coli* chromosome by the process of interrupted mating. This technique can not be used in *Streptomyces*, however, gross chromosomal linkage mapping is possible, by analysing a series of conjugation and recombination events (Hopwood, 1972). Chromosomal sequences from one strain may appear as free plasmids in others. For example the SLP1 sequence from *S.coelicolor* is a plasmid in *S.lividans* (Bibb et al., 1981). Matings allow the study of conjugative plasmids by crossing a well-characterised strain (plasmid-free *S.lividans*) and the strain to be tested. This method is used to locate new pock (lethal zygois) forming plasmids, which most *Streptomyces* conjugative plasmids can produce. These pocks are markers of genetic recombination and a useful tool for genetic analysis. Hopwood et al. (1984) removed two endogenous plasmids from *S.lividans* strains TK23 and TK24 which were more useful because the additional antibiotic resistance (spectinomycin or streptomycin)

allows counter selection after crossing.

Recombinant DNA techniques in *Streptomyces* genetics preferentially use plasmids to clone large fragments of DNA. Plasmids rather than phages are employed to clone biosynthetic genes. Phages are utilised if the DNA being cloned is for use in genetic studies with collections of mutants. Phages are also used if the DNA is to be introduced into a species with poor protoplasting properties.

1.6.2 Transformation and transfection

The method for producing competent cells induced by cold shock and calcium treatment used in plasmid-mediated cloning in *E.coli* is not possible for *Streptomyces*. The basis for all *Streptomyces* transformations, including the ones in this project, is high frequency protoplast formation in polyethylene glycol (PEG) (Bibb et al., 1978). Plasmids are taken up readily by protoplasts. Efficiencies of 10^6 - 10^7 of *S.lividans* and *S.coelicolor* per μg plasmid DNA are common. Despite these high frequencies Okanishi et al., (1983) listed many parameters that influence transformation frequency. These include, growth phase of mycelium, temperature at which mycelium and protoplasts are incubated, protoplast concentration, dryness of regeneration plates and washing the protoplasts to remove nucleases liberated by the protoplasts. In addition to these factors Kieser (1984) found that ribonuclease added to plasmid preparations

reduced the frequency of protoplast regeneration and transformation efficiency.

When transfecting protoplasts the same conditions used for optimal transformation yields 10^5 plaques per μg of ϕC31 DNA.

1.6.3 Mutation in *Streptomyces*

The procedure of mutagenesis has aided the understanding of gene organisation structure and regulation. Three agents are used routinely, ultraviolet radiation (UV), long wave UV in the presence of 8-methoxysporalen and N-methyl-N'-nitro-N-nitrosoguanidine (NTG). Random chemically induced mutation is a widely applied and successful genetic procedure improving the antibiotic productivity of *Streptomyces*. Despite the widespread use of mutation relatively little is known of the fundamental mechanism of mutation and repair in *Streptomyces*. NTG is an effective mutagen and in other organisms caused lesions in the DNA and additional clustered mutations. Randazzo et al., (1973) used the clustering of NTG mutagenesis to induce mutations within particular chromosomal regions of industrial significance.

8-methoxysporalen is often chosen for precisely the opposite reason. It induces mutations randomly in different genes (Scott and Alderson, 1971) because it sensitises the DNA to UV light. However, despite the alleged ability of methoxysporalen to cause random

mutation it is not used routinely in the laboratory. This is a reflection of the widespread use of short wave UV radiation due to it being convenient and relatively safe. The *S.lavendulae* 'DNJ' producers used in this project were mutated using short wave UV and screened for non-production of NOJ using a bioassay (Trew, 1992). Experiments involving the feeding of nojirimycin to these blocked mutants and the significance of the results are detailed in Chapter 3.

Some of the other procedures used in this project do cause mutations. For example, protoplast formation, fusion and regeneration are all associated with genetic instability. A detailed investigation into the loss of plasmid DNA was undertaken by Hopwood et al. (1983) and the results indicated that formation and regeneration of protoplasts caused the plasmid deletions. The possible influence of this form of mutation is examined in further detail when the results of the cloning are analysed in Chapter 4.

1.6.4 Production of gene banks.

A strategy to clone biosynthetic pathways frequently utilises high copy number vectors. The vector pIJ702 used in this project was derived from the broad host range, multicopy plasmid pIJ101. A 1.55kb *Bcl* I generated fragment was sub-cloned into the multicopy plasmid pIJ350 (Katz, 1983). The fragment can enter two *Bcl* I sites in either of two directions to generate 4 hybrid plasmids pIJ702-pIJ705. pIJ702 has 40-300

copies per chromosome and carries a thiostrepton gene for vector selection. Insertion into single sites *Bgl* II, *Sph* I and *Sst* I abolished melanin synthesis and allowed selection of recombinants (Fig 1.2).

pIJ702 has been used to clone uracil and α -amylase on whole DNA inserts of 12kb and 6kb respectively (Feitelson, 1983). Whole DNA inserts of 6kb and 7.5kb were also used to clone puromycin (Vara et al., 1985) and kanamycin (Nakano et al., 1984) into a heterologous host. Similarly, Kendal and Cullum (1984) shotgun cloned fragments of *S.coelicolor* into pIJ702 plasmids and found the agarase gene located on a 1.9kb segment. Fragments of between 10kb-15kb have also been used to complement mutants of undecylprodigiosin. Mutants of *S.coelicolor* were obtained using ultra violet (UV) radiation. These were arranged into complementation groups A-E by cosynthesis experiments. The wild type *S.coelicolor* was digested with *Bcl* I and cloned into the *Bgl* II site of pIJ702. The blocked mutants were transformed with these recombinant pIJ702. A 4.72kb fragment restored the antibiotic production.

Having cloned DNA present at high copy number may lead to the translation of toxic metabolic intermediates, especially when cloning large pieces of DNA. It is more advisable to use low copy number plasmids and most are derived from either SLP1.2 or SCP2. SLP1.2 has produced many vectors including pIJ61 which has only 4 or 5 copies per cell. pIJ61 is an autonomous conjugative plasmid with a neomycin resistance gene from *S.fradiae*

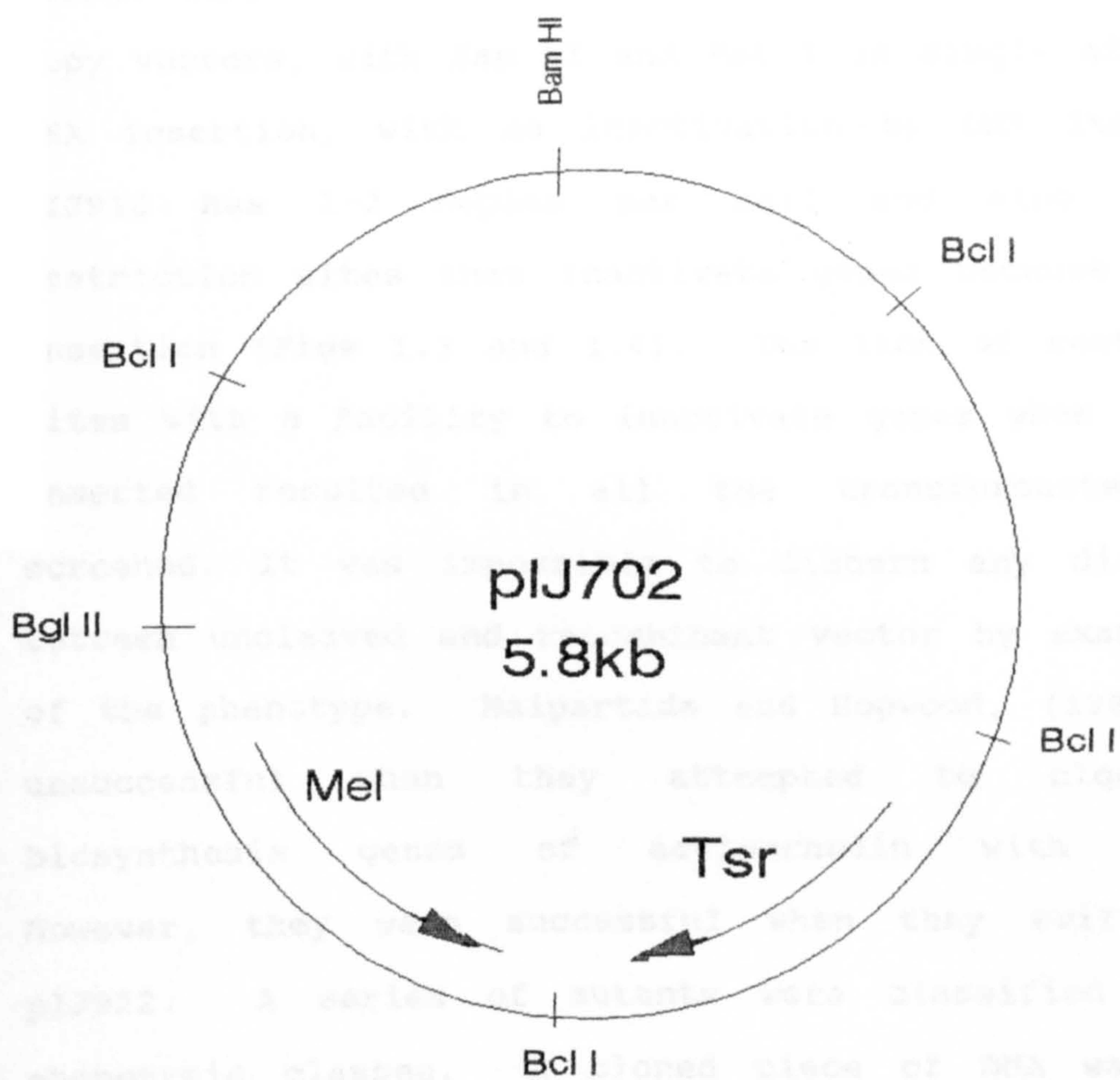
Fig 1.2

A MAP OF THE HIGH COPY NUMBER VECTOR pIJ702
SHOWING THE RESTRICTION SITES FOR Bgl II, Bam HI
AND Bcl I.

Mel – Melanin Biosynthesis

Tsr – Thiostrepton Resistance

Modified from Hopwood et al. (1985)



and a thiostrepton resistance gene from *S. azureus*. It has single insertion sites for DNA into *Bam* HI or *Pst* I which inactivate neomycin.

The other main source of vectors was the plasmid SCP2 from which pIJ913, pIJ916, pIJ943, pIJ922, pIJ940 and pIJ941 were all derived. pIJ922 and pIJ940 are single copy vectors, with *Bam* HI and *Pst* I as single sites for DNA insertion, with no inactivation by DNA insertion. pIJ916 has 1-2 copies per cell and also has no restriction sites that inactivate genes because of DNA insertion (Figs 1.3 and 1.4). The lack of restriction sites with a facility to inactivate genes when DNA was inserted resulted in all the transformants being screened. It was impossible to discern any difference between uncleaved and recombinant vector by examination of the phenotype. Malpartida and Hopwood, (1984) were unsuccessful when they attempted to clone the biosynthesis genes of actinorhodin with pIJ702. However, they were successful when they switched to pIJ922. A series of mutants were classified into 7 phenotypic classes. A cloned piece of DNA was found which complemented mutants of all classes. Using pIJ922 cloned pieces of DNA in excess of 30kb could be efficiently transformed into *Streptomyces* protoplasts. Another SCP2 derivative, pIJ916 was used by Ikeda et al., (1984) to clone DNA fragments of 10kb - 30kb. These fragments were used to complement glucose kinase (*glk*⁻) mutants of *S. coelicolor*.

Fig 1.3

A MAP OF THE LOW COPY NUMBER VECTOR pIJ916
SHOWING THE RESTRICTION SITES FOR Bgl II,
AND Bcl I.

Tsr – Thiostrepton Resistance

Modified from Hopwood et al. (1985)

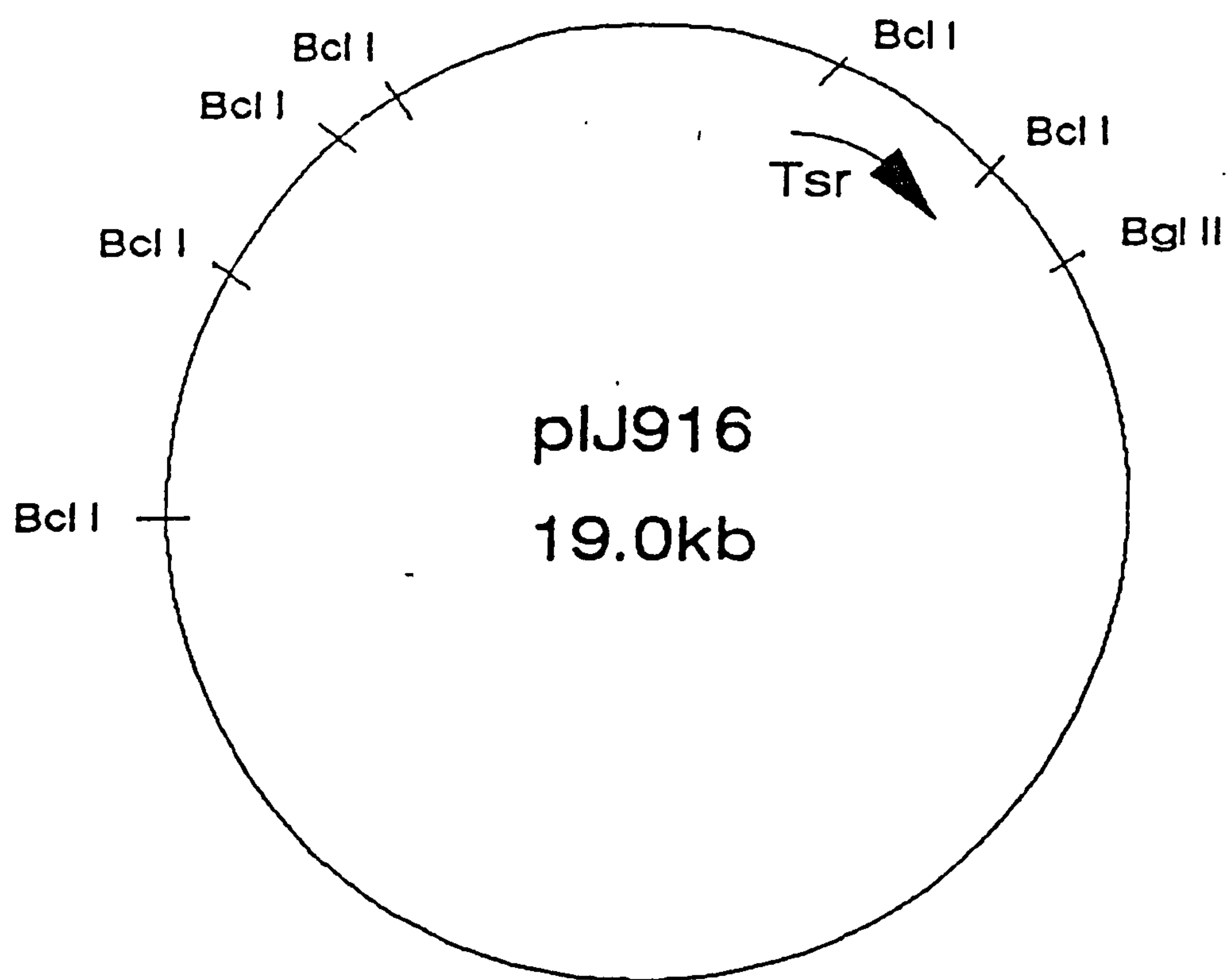


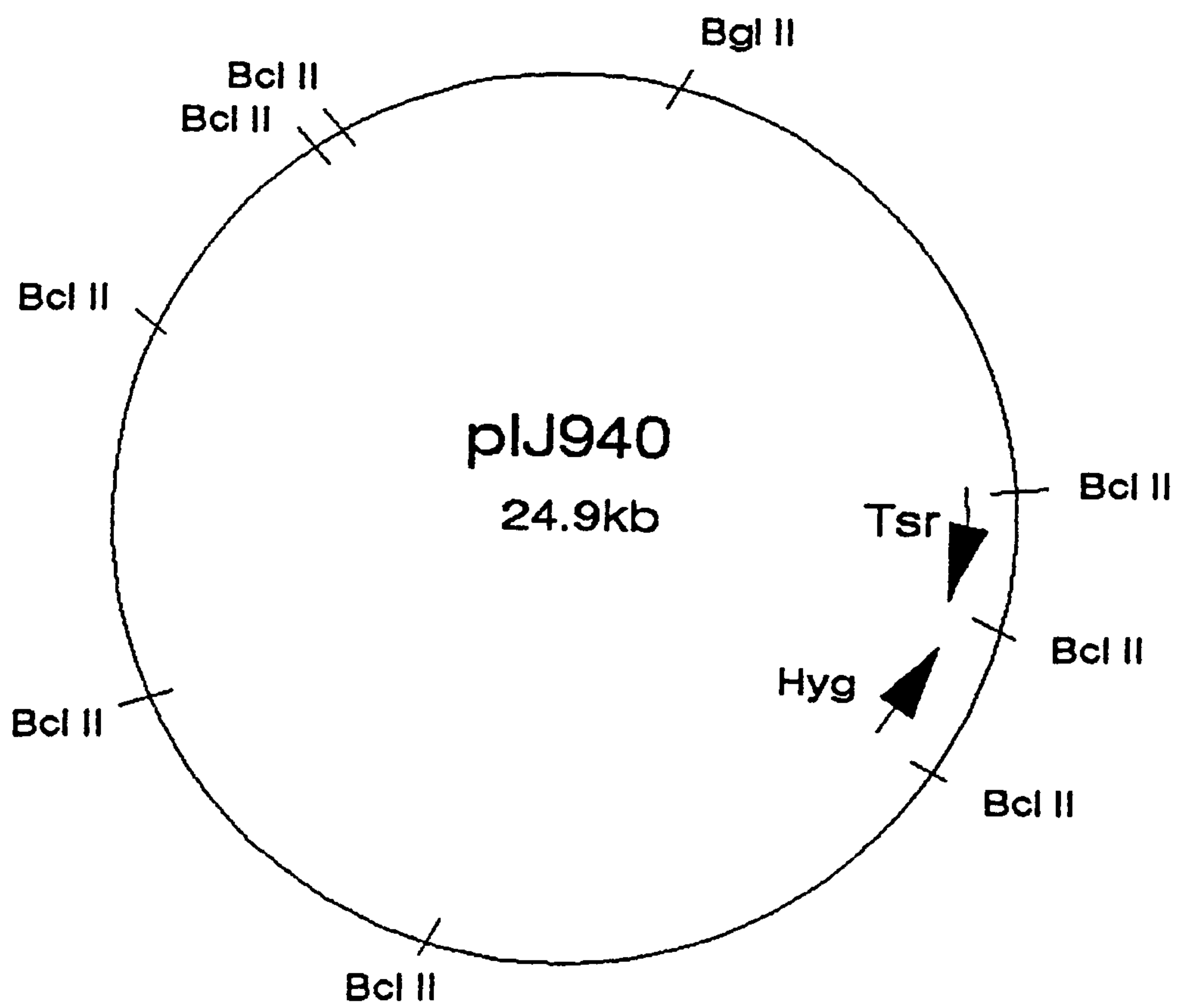
Fig 1.4

A MAP OF THE LOW COPY NUMBER VECTOR pIJ940
SHOWING THE RESTRICTION SITES FOR Bgl II
AND Bcl I.

Tsr - Thiostrepton Resistance

Hyg - Hygromycin Resistance

Modified from Hopwood et al. (1985)



The main alternative cloning vector, instead of using plasmids, is the use of either generalised or specialised transducing phages.

Generalised transduction occurs because the DNA of the host cell becomes severely degraded during phage infection. Random DNA fragments the size of the phage genome can become incorporated into phages. However no generalised transducing phages are used as cloning vectors in *Streptomyces*. Specialised transduction is a feature of lysogenic (temperate) phages. A lysogen is formed when the phage enters the chromosome at a specific site. The phage DNA is then reproduced along with the chromosomal DNA. The lysogenic state can be disrupted and the phage genome excised in a burst of infection. Phage excision is usually precise but it may also excise adjacent cellular genes and incorporate them into the phage genome. Only genes close to the integration site are transducible. ϕ C31 is a temperate phage forming plaques on two thirds of *Streptomyces* species. The phage enters the genome at a specific att site and is maintained by a repressor C gene. Phage and donor DNA are cut with restriction enzymes and ligated in vitro. Each phage contains a different fragment of donor DNA. After transfection of protoplasts and plating onto an indicator strain, recombinant molecules are recognised by plaque formation. These plaques can be picked off in the presence of antibiotic selection. Glucose kinase was cloned from a 3.5kb DNA insert using ϕ C31 KC515 (Ikeda et al., 1984). This fragment restored all *glk*⁻ mutants to

the wild type phenotype.

Mutational cloning is a variation of cloning with phages and was developed by Chater and Bruton (1985) to clone the methylomycin (mmy) biosynthesis genes from the giant linear plasmid SCP1. Phage DNA containing viomycin resistance was digested with *Pst* I. This was ligated to donor DNA from *S.lividans* which had also been digested with *Pst* I. The resultant recombinant phage DNA was transfected into protoplasts and plated out on the indicator strain. Plaques were examined for viomycin resistance to show that phage DNA was present. Fourteen percent of these contained SCP1 genes, a direct result of the plasmid amounting to about 14% of the total cellular DNA. Attention then focused on the methylomycin producers. Low mmy producers could be a result of inactivation by homologous DNA inserts that disrupt the mmy biosynthetic pathway. Once homologous DNA had been found, restriction analysis and southern hybridisation led to the determination of a biosynthesis pathway of 20kb. This technique is most useful if a large amount of DNA is to be transcribed, for example when cloning antibiotic biosynthetic genes.

Cosmids are hybrid vectors constructed by recombinant DNA techniques. They are plasmids containing a lambda DNA insert, a minimum of 38kb can be packaged within the cos site of a cosmid. The use of cosmids is becoming increasingly common because traditional problems of expression in *Streptomyces* are being overcome. A useful cosmid vector was constructed by McHenney and

Baltz (1991). They cloned the (*hft*) segment of bacteriophage FP43 into the *Sph* I site of pIJ702 and termed this plasmid pRHB101. The cloned DNA contained an origin for headful packaging or *pac* site. Furthermore a 1.6kb *Kpn* I fragment containing the *ermE* gene for expression of erythromycin resistance was cloned into the cosmid. Transduction into all *Streptomyces* species tested was at a level similar to those observed with the parent plasmid. Additionally, lysates of FP43 prepared on *S.griseofuscus* containing pRHB101 transduced some *Streptomyces* species which would restrict bacteriophage FP43. Two other *Streptomyces* species known to contain restriction enzymes that cut pRHB101 DNA were also transduced.

Gaudreau et al. (1992) examined the nature of the *cos* site by cloning the ends of a bacteriophage from a 488bp fragment. Many shuttle *E.coli-Streptomyces* cosmids exist but with the exception of the cosmid constructed by McHenney and Baltz (1991) all contain the lambda *cos* site and only package DNA inserts into *E.coli* systems. Transduction has been demonstrated with the *cos* ends of actinophage R4 and ϕ C31, but there was only the one *in vivo* and *in vitro* packaging system available. A shuttle cosmid pOJCOS305 was constructed which contained all the *cos* ends of phage JHJ-1 and all the sequences to package DNA *in vivo*. Plasmid pOJCOS305 transformed in *S.erythraea* was transduced upon infection with particles of phage JHJ-1. The lysate was then used to transfer donor DNA to other recipient strains.

The most well known case of the application of cosmids towards cloning antibiotic biosynthesis genes was the cloning of the erythromycin biosynthetic pathway (Tuan et al., 1990). The constructed plasmids replicated in *E.coli* and *S.lividans*. A total genomic library of *S.erythraea* was constructed and plasmids were used to restore blocked mutants of erythromycin biosynthesis. The library was probed with these DNA fragments containing the resistance genes. This allowed the 5kb region responsible to be mapped and southern blots showed it to be completely internal to the biosynthesis genes.

An alternative approach using cosmids is to make a cosmid library and probe for biosynthetic genes using labelled biosynthetic genes from other species. Smith et al. (1990) isolated a cosmid clone containing linked β -lactam biosynthetic genes from a gene library of *Flavobacterium* spp. The DNA was located by cross hybridisation of corresponding DNA from *S.clavuligerus*, *P.chrysogenum* and *Aspergillus niger*. They all contain the α -(L- α -amino adipyl)-L-cysteiny-D-valine synthetase (ACVS). This is the initial enzyme involved in penicillin biosynthesis and were all linked to other β -lactam genes that constituted part of the gene cluster.

The use of shuttle vectors was a strategy that became more of a possibility, once initial problems of incompatibility, using the high and low copy number plasmids had been identified.

Most shuttle vectors are constructed by fusing two plasmids and so are traditionally quite large, for

example Fayerman (1986) described the use of shuttle vectors to clone arginine biosynthesis from *S.cattleia* to *E.coli* using pBR322. Shuttle vectors were also used to clone glycerol metabolism from *S.coelicolor* to *E.coli* with pBR327 (Smith and Chater, 1986). Recently there have been developments to reduce the size of these vectors. Neesen and Volckaert (1989) cloned a small *E.coli* replicon containing chloramphenicol into pIJ702. The vector contained many unique sites for cloning but was only 5.25kb. The differences in transformation frequencies were dependant on whether plasmid DNA was isolated from *E.coli* GM119 or *E.coli* HB101. Shuttle vectors can also transfer DNA by inter-generic conjugation, for example Mazodier et al. (1989) used *E.coli-Streptomyces* shuttle vectors pPM801 (14.4kb) and pPM803 (10.5kb) and produced exconjugants in *S.lividans*, *S.pristinaespiralis* and *S.viridochromogenes*.

Biermann et al. (1992) made 14 shuttle vectors, which were plasmid constructs of use in cloning experiments. All the vectors contained the origin of transfer (*oriT*) fragment from the incompatibility factor (*IncP*) plasmid RK2. Gene transfer was via conjugal transfer and transfer functions were supplied by the *E.coli* donor strain. Five vectors were non-replicating and were used for gene disruption and gene replacement experiments. The remaining 8 contained replication functions and could exist as multicopy autonomous plasmids. For example, pKC1139 is only 6.5kb and yet is capable of accepting inserts of DNA up to 35kb.

1.7 *Streptomyces* Phages and their interaction with restriction systems.

KC301 is a derivative of ϕ C31 modified by the insert of a thiostrepton resistance marker. The parent phage ϕ C31 can lysogenise and/or form plaques on two thirds of *Streptomyces*. The prophage integrates into the chromosome at a specific att site and is maintained by a repressor C gene of the phage (Smith et al., 1992). Phage KC301 is maintained in the host via the same mechanism and still contains the same cohesive ends as ϕ C31. Restriction-modification systems have experimental significance within the *Streptomyces*, because restriction systems may cleave introduced DNA from inter-specific gene transfer, protoplast fusion or cloning. KC301 was used to test the restriction-modification system between *S.lavendulae* and *S.lividans*. A typical restriction-modification system was exhibited between *S.levoris* and *S.hygroscopicus* using the phage SH5 (Klaus et al., 1981). The phage titre was totally dependant upon the previous *Streptomyces* strain in which the phage originated. If the strain was unchanged the phage titre was maintained. However, phage numbers were reduced by 10^4 with the introduction of an alternative host. Modification involved methylation of restriction sites which then prevented the enzyme restricting the DNA at that site. The system was further complicated by modification mutants occurring even when no modification

system was present. In the above example SH5 DNA has more than 25 restriction sites for *Shy* I with an average restriction coefficient of 3×10^{-2} per site. The DNA of *S.hygroscopicus* should be totally degraded but complete degradation was prevented by restriction-minus modification-plus (r- m+) mutants.

The restriction-modification system has also had some evolutionary influence. If the phage does not contain restriction sites degradation can be avoided. Kruger and Bickle (1983) site evidence for this in *Bacillus* phages. *Streptomyces* phages also provide numerous examples of the lower incidence of restriction sites, for example the number of restriction sites for *Sst* I, *Sal* PI and *Sri* I in their DNA. DNA in excess of 40kb and a GC content >66% should have 60 sites where G-C pairs account for 6 of the 6 pairs. Fifteen sites should contain 4 G-C pairs of the 6 and an estimated 4 sites for 2, G-C pairs out of 6. Only the number of restriction sites of *SalG* I, *Sph* I and *Sca* I match the estimated number in phage DNA examined to date and these are comparatively rare enzymes so selection pressure against possession of such sites is much less. Lambda phage contained recognition sites for all the enzymes at the expected frequency.

1.8 Screening

Sixty percent of naturally occurring antibiotics are derived from *Streptomyces*, which produce representatives of all antibiotic classes (Okami and Hotta, 1988). Current efforts are focused on the increasing difficulty of finding new antibiotics as the number of known antibiotics increases.

Screening procedures usually involve an initial isolation stage, assaying the isolates for the antibiotic and finally characterising the antibiotic chemically. Since the late 1960's the antibiotic assays have improved and can detect target antibiotics, even at low concentrations. For example the use of hypersensitive mutants or enzyme assays to detect β -lactam antibiotics. Okamura et al. (1979) used *Comamonas terrigena* to screen for inhibitors of cell wall synthesis. Other assays assessed the specificity against β -lactamase producing strains in the presence of a β -lactam antibiotic (Brown et al., 1976). The screening demonstrated that many taxa were capable of β -lactam production. Screens became target-directed and the selection of the organisms was used in combination with target directed assays. One of the best examples was an organised programme by Hotta et al. (1983a) to detect aminoglycoside antibiotics. Actinomycetes were isolated resistant to an aminoglycoside. When their resistance to a wide range of aminoglycosides was characterised it was revealed that

taxonomically diverse actinomycetes with multiple resistance were widely distributed in nature. The use of antibiotics enhanced isolation of strains with aminoglycoside resistance. Furthermore these same isolates have a much higher rate of antibiotic production (Hotta et al., 1983b). Different aminoglycoside resistance patterns were signals or phenotypic markers for production of different antibiotics (Takahashi et al., 1986). There have also been screening programmes designed for glycopeptides. A similar investigation by Phillips et al. (1992) examined DNA sequences hybridising with different resistance genes and antibiotic production probes. The resultant homologies were clustered and examined for phenotypic antibiotic resistance. Producers of certain classes of antibiotic were clustered and these same strains tended to be producers of other antibiotics. For example hybridisation with the aminoglycoside phosphotransferase (*aphD*) probe for streptomycin resistance was correlated with aminoglycoside production.

A more fundamental aspect of screening is the medium employed for production of the antibiotic. Different culture media favour the production of certain antibiotics. Aminoglycoside antibiotics were preferentially produced when the medium contained starch and soybean meal as carbon and nitrogen sources. Pyrrolnitrin was discovered when this medium was employed (Arima et al., 1965). Other antibiotics were only discovered when grown in medium containing unusual concentrations of nutrients. Thiopeptin was discovered

when the medium contained 100-200mM inorganic phosphate (Miyairi et al., 1970). The medium used was complex, since this medium would contain all the relevant nutrients for a wide spectrum of antibiotic production. However, aplasmomycin was discovered when a complex medium was diluted sixteen fold (Okami et al., 1976).

The physical nature of the medium has also been shown to be important with many strains producing antibiotics on solid media but rarely in liquid media. For example, Omoto et al. (1979) demonstrated that *Streptomyces halstedii* produced N-carbamoyl-D-glucosamine on solid agar but production was absent in liquid culture. Fragmentation accounted for the lack of production in liquid systems and a non fragmenting mutant was employed for commercial production.

Many new antibiotics have been discovered by feeding specific precursors to various antibiotic producers. An example of this was the production of chimeramycins by feeding precursors to a spiramycin-producing strain (Omura et al., 1983). Similarly, *Penicillium chrysogenum* accumulates penicillin G in the medium when fed phenyl acetic acid. Biosynthetic penicillin can then be made from penicillin G by chemical synthesis incorporating a variety of products into the side chain (Demain, 1981).

Other biosynthetic enzymes can convert existing antibiotics to new antibiotics. Kanamycin was converted to amikacin (Cappelletti and Spagnoli, 1983) using a butirosin-producing strain of *Bacillus.circulens*.

1.9 Application of streptomycete metabolites

Streptomyces products that have diverse applications. Commercial production of enzymes is financially the second most important after antibiotic production (Peczynska-czoch and Mordarski, 1988). Industrial methods of enzyme production can give high yields and be cost effective. Enzymes are used in a range of industrial processes including: food processing, detergent manufacture, medical therapy and the textile and pharmaceutical industry.

D-xylose isomerase (formerly glucose isomerase) is one of the most important enzymes produced by the actinomycetes. The enzyme can be obtained from *Actinoplanes missouriensis*, *Streptomyces olivaceus*, *Streptomyces albus* and several other *Streptomyces* species. The enzyme produces D-fructose from D-xylose, has a pH optimum of 7-10 and is stable at temperatures of 60-85°C. Other *Streptomyces* strains are responsible for production of many enzymes. For example, *S. griseus* excretes many protease enzymes into the culture during the logarithmic phase of production. This solution is termed pronase and contains several protease enzymes and peptidases which can be further purified.

In medical therapy enzymes are involved in diagnostic tests of blood serum. Cholesterol oxidase provides a rapid and sensitive determination of cholesterol, while urate oxidase indicates uric acid (Everse et al., 1979).

Penicillin amidases from *Mycobacterium*, *Nocardia* and

Streptomyces are used in the industrial production of 6-aminopenicillanic acid after hydrolysis of penicillins (Vandamme, 1974).

Semi-synthetic penicillins and cephalosporins can be synthesised from D-amino acids such as D-phenylglycine. Aminocyclases are used to synthesise these optically active amino acids (Szwajcer et al., 1981). These two enzymes are examples of a host of enzymes now used in biotechnology. Enzymes from every enzymatic classification including oxidoreductases, transferases, hydrolases, transferases and lyases are synthesised and exploited.

As well as the enzymes themselves, many enzyme inhibitors have been identified to be of microbial origin (Umezawa, 1988). These compounds are usually small molecular weight compounds and up to 1988 only 60 had been reported (Umezawa, 1988). They are not antibiotics and most have no significant microbial activity. Umezawa, (1972) reported that leupeptin is a protease inhibitor produced by eleven species of soil *Streptomyces*, yet it has no obvious biological function. The nitrogen-containing alkaloids of which DNJ is a member are a family of enzyme inhibitors.

Some of these inhibitors are used primarily as an animal foodstuff additive. For example tylosin from *S. fradiae* and monensin from *S. cinnamomensis* are used as animal growth promoters, with some secondary protection against infection.

In the field of medicine, filtrates are screened for

anti-tumour or cytotoxic properties. These drugs are mainly anthracyclines for example daunomycin and mitomycin C. Screens specifically target inhibition of tumour growth *in vivo*, or target tumour cell growth *in vitro*. During the course of screening for alkaline phosphodiesterase inhibitors Glyo-II from a *S.griseosporus* culture inhibited glyoxalase. Glyo-II lacked antibacterial activity at concentrations of 100µg/ml but inhibited Yoshida rat sarcoma cells at concentrations of 18µg/ml in tissue culture (Sugimoto et al., 1982)

Apart from direct inhibition of tumours other products are useful for chemotherapy because they have an immunity-enhancing effect. Bestatin from *S.olivoreticuli* (Umezawa et al., 1976) and forphenicine from *S.falvoviridis* (Aoyagi et al., 1978) both enhanced the immune response in mice by inhibiting hydrolytic enzymes on the cell surfaces. Bestatin, forphenicine and forphenicinol all suppress both the generation and action of suppresser cells against delayed type hypersensitivity. They also intensified cell mediated immunity, T cell proliferation and natural killer cell activity. The drug FK-156 has attracted recent attention as an immunity augmenting product. The drug was found to exhibit anti-tumour activity in animals (Izumi et al., 1983).

The phagocytic index was enhanced as well as humoral antibody formation when 0.01-10mg/kg was administered. The drug was also relatively non-toxic, the LD 50 was more than 1g/kg when administered intravenously to mice.

1.10 The nitrogen-containing alkaloids and their biosynthesis.

1.10.1 Nojirimycin

Nojirimycin (NOJ) 5-amino- 5-deoxy-D-glucopyranose, was first isolated from a *Streptomyces* strain in 1966 and was the first naturally occurring 5-amino sugar to be discovered. It was originally termed a heterose with the hetero atom (oxygen) being replaced by a sulphur or nitrogen atom.

NOJ has pronounced biological activity. Ishida and Shomura (1966) used *Shigella* as a screen to detect inhibitors including NOJ. Two other species of *Streptomyces* also produced NOJ and further testing showed it to have activity against *Sarcina lutea*, *Shigella flexneri* and *Xanthamona soryzae*. The antibiotic was active enough to prevent rice blast in pot tests. The NOJ was isolated as a weakly basic, water soluble solid from fermentation of *S.rosechromogenes*, *S.lavendulae* and *S.nojiriensis*. The inhibitor could not be removed with an organic solvent so it was bound to strongly acidic cation exchangers and then eluted with aqueous ammonia.

NOJ is not only produced by *Streptomyces*. Puls et al. (1980) described production of NOJ from several strains of *Bacillus* including *B.polymyxa*, *B.subtilis*, *B.amyloliquifaciens* and *B.niger*.

An extensive examination of the inhibitory properties

of NOJ found it inhibited α -glucosidase (the molar concentration required to give 50% inhibition was 1×10^{-5} M.) approximately ten times more effectively than β -glucosidases (1×10^{-6} M.) NOJ was ineffectual against inhibiting the exo-enzyme glucoamylase. However, NOJ was a more effective inhibitor than D-glucono-1,5-lactone, the other inhibitor assayed. The enzyme α -trehalase was also examined, although DNJ was not included in the study, the enzyme was relatively insensitive to the inhibitors tested (Reese et al., 1971).

Castanospermine or 1,6,7,8-tetrahydroxyoctahydroindolizine was first isolated by Hohenschutz et al. (1981) from the chestnut seeds of an Australian tree legume. Castanospermine is an indolizidine alkaloid and is a bigger molecule than either DNJ or NOJ.

The effect of swainsonine was initially seen by nineteenth century Australian settlers. Their cattle developed neurological and behavioural abnormalities after eating small pea-like plants of the legume genus *Swainsona*. The condition was very similar to a hereditary condition that occurs in man and other animals called mannosidosis. The disease is a result of the loss of α -mannosidase and leads to an accumulation of ungraded mannose-containing oligosaccharides in the lysosomes. The *swainsonia* legumes were found to contain a mannosidase inhibitor which was isolated (Colegate, et al., 1979) and termed swainsonine after the genus of legumes. Swainsonine has since been identified from other

legumes for example *Oxytropis* and *Astragalus* and also from the fungi *Rhizoctonia leguminicola*.

1.10.2 Deoxynojirimycin

Deoxynojirimycin (DNJ), 1,5 - dideoxy - 1,5 - imino - D- glucitol, was isolated during routine screening (Yagi et al., 1976). It was isolated from a mulberry bush *Morus spp* and so the antibiotic was termed moranoline. This compound was later found to be already in existence under the name deoxynojirimycin. It was derived from the related compound nojirimycin (NOJ) by catalytic hydrogenation over a platinum catalyst. Under the rules of nomenclature the name moranoline was dropped and now only DNJ is used. DNJ is a structural analogue of glucose in which the ring oxygen is replaced by nitrogen and the hydroxyl group at the C1 carbon atom is missing. The similarity of the structure of DNJ and its oxidised compound NOJ to glucose is shown in Fig 1.5.

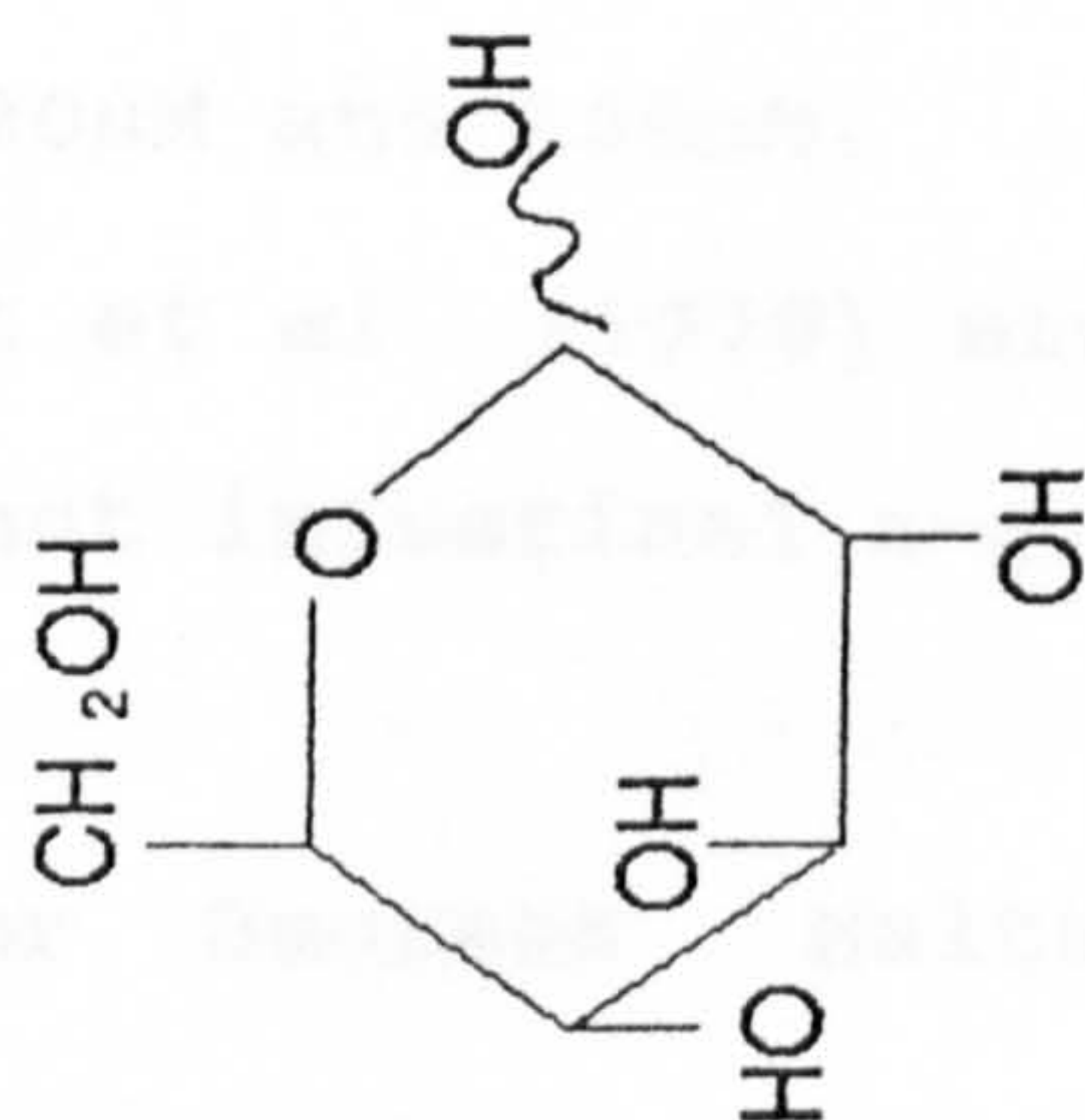
DNJ has a variety of applications including an antihyperglycaemic agent, an insect antifeedant, and an inhibitor to the spread of cancers. There is particular interest concerning the ability of DNJ to inhibit synctium formation and the increase of Human

Fig 1.5

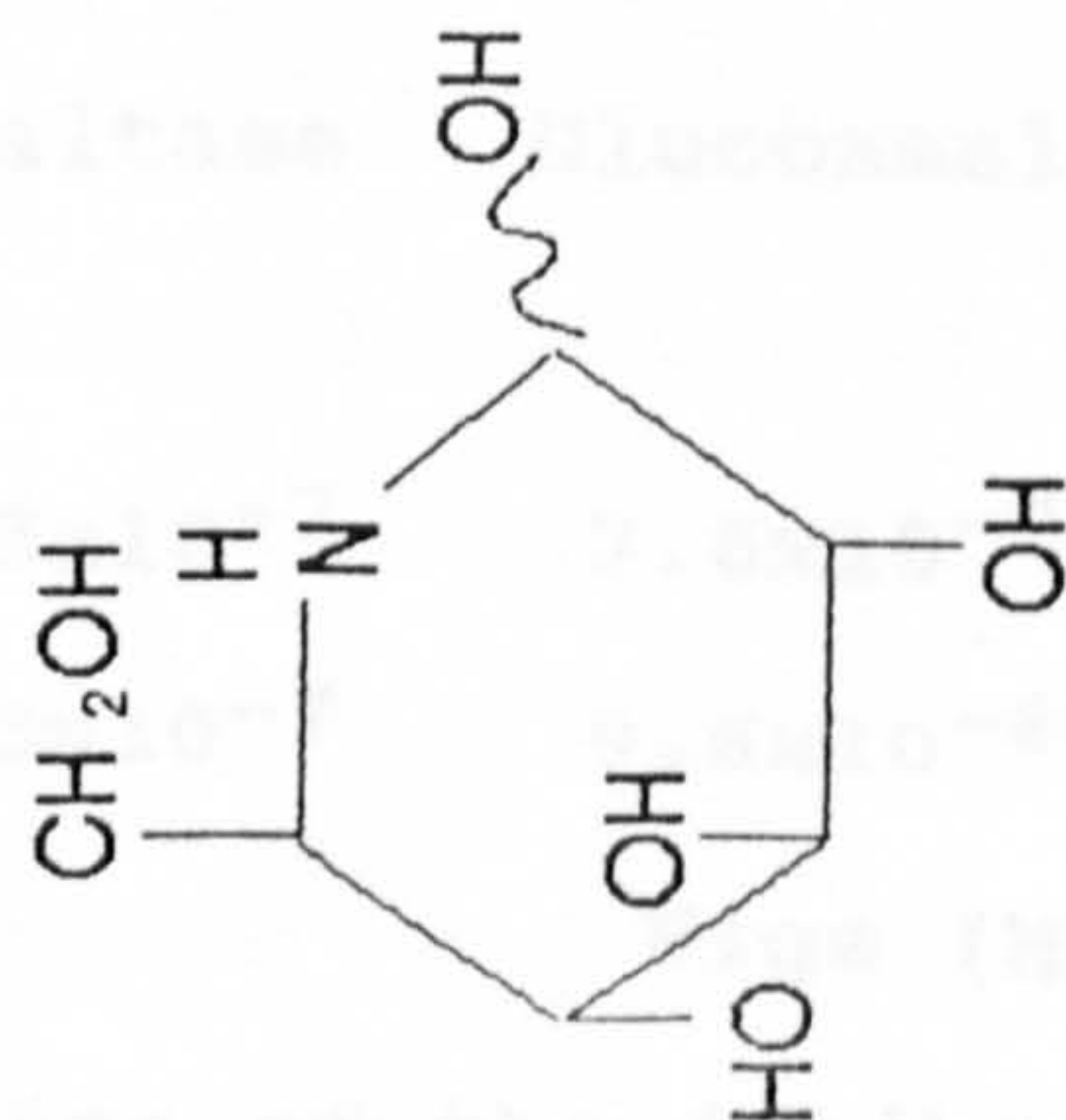
DEOXYNOJIRIMYCIN A STRUCTURAL ANALOGUE
OF GLUCOSE.

The oxygen atom in the glucose molecule is replaced in DNJ by a nitrogen atom and a hydroxyl group is removed.

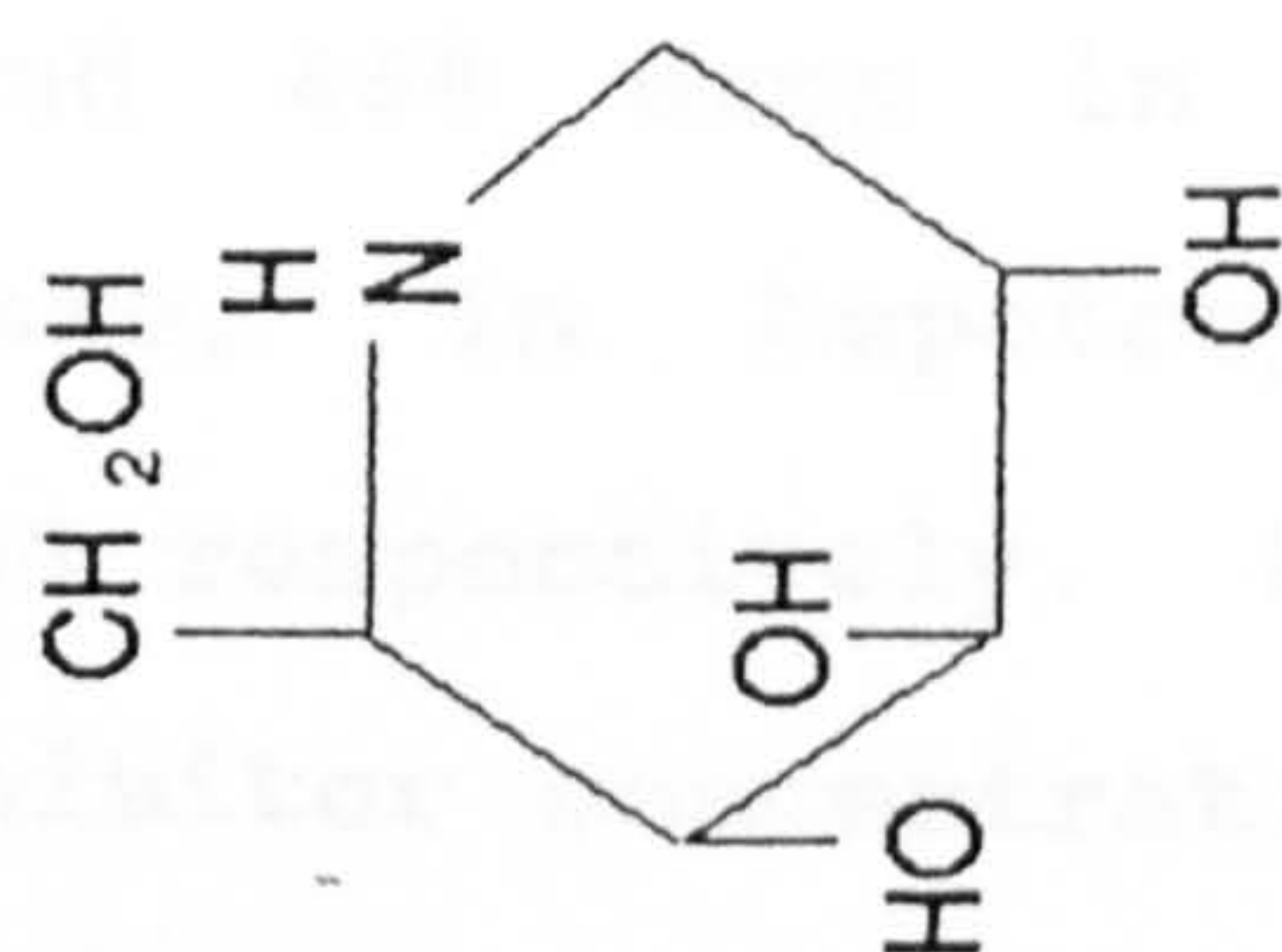
GLUCOSE



NOJIRIMYCIN



DEOXYNOJIRIMYCIN



Immunodeficiency Virus (HIV). The first useful biological activity for DNJ was found, shortly after its discovery. Doses of 10mg-100mg kg⁻¹ partially or totally inhibited the postprandial rise in blood sugar when administered to rats with 2g kg⁻¹ sucrose (Yagi et al., 1976) . A patent was filed as an antidiabetic agent.

These medical applications were further developed by Bollen et al. (1988). Two chemical derivatives were formulated by Bayer, BAY1099 and BAY1248. They were hyperglycaemic drugs and had a direct effect on the liver. There was a 70%, 60% and 45% drop in the conversion of glucagon to glucose, in hepatocytes inhibited by BAY1248, DNJ and BAY1099 respectively. Half maximal effects were observed at inhibitor concentrations between 20µM and 100µM.

Schmidt et al. (1979) studied the inhibition of DNJ and NOJ against intestinal α-glucosidase.

Inhibitor	Sucrase	Maltase	Isomaltase	Glucoamylase
NOJ	5.6x10 ⁻⁷	1.7x10 ⁻⁶	2.5x10 ⁻⁷	7.6x10 ⁻⁷
DNJ	2.2x10 ⁻⁷	1.3x10 ⁻⁷	1.3x10 ⁻⁷	9.6x10 ⁻⁸

Figs (M.)

More detailed studies of the kinetics of the inhibition by DNJ, NOJ and acarbose (a synthetic oligosaccharide containing nitrogen in place of the ring oxygen), against intestinal sucrase showed all these compounds to be competitive inhibitors (Hanozet et al., 1981).

All the above compounds were competitive inhibitors and had K_i values within the range 10^{-7} M and 10^{-8} M This indicated they were powerful inhibitors of intestinal sucrase.

Table 1.1 The K_i values of selected inhibitors of sucrase.

Inhibitor	pH	K_i (μ M.)
Acarbose	6.8	0.47 + 0.05
NOJ	6.8	0.127 + 0.017
NOJ	4.5	0.33 + 0.03
DNJ	6.8	0.032 + 0.004
DNJ	5.85	0.115 + 0.005

The inhibitory affect of DNJ against α - and β -glucosidases was extensively studied (Evans et al., 1985).

This information allowed these glucosidases to be used as targets in screens to detect other compounds with similar inhibitory properties. Atsumi et al. (1990) used p-nitrophenyl - β -D-glucopyranoside to screen 1000 strains of bacteria and mushrooms. A mushroom *Phellinus* spp. produced a substance which inhibited almond β -glucosidase. The extracted substance was named cyclophellitol and it was hoped this would inhibit HIV in a similar fashion to DNJ and castanospermine. The IC_{50} (concentration required to inhibit 50% of the reaction)

of cyclophellitol was $0.8\mu\text{g/ml}$. The IC_{50} for DNJ was $30\mu\text{g/ml}$ and it was $12\mu\text{g}$ for castanospermine. These results clearly indicate the increased inhibitory properties of cyclophellitol against α -glucosidase. The inhibition of HIV by DNJ is potentially the main medical application. To appreciate how DNJ and its structurally related analogues inhibit HIV infection it is important to understand the normal cycle of infection. The env gene of HIV-1 encodes the intracellular precursor glycoprotein gp160 which is produced in infected cells. This is cleaved proteolytically to form gp120 the external viral glycoprotein and gp41 a transmembrane protein which anchors gp120 to the envelope. These glycoproteins mediate attachment of virions to glycosylated cell surface receptor molecules (CD4 antigens). A hydrophobic domain at the amino terminus of gp41 is thought to be responsible for the membrane fusion events in HIV infection and syncytium formation. Expression of viral gp120 encourages cell-cell fusion and cell death. This leads to an overall depletion of T cells with the CD4 component. Gp120 is very glycosylated, 50% of the molecular weight consists of N-linked carbohydrates containing high mannose and complex hybrid oligosaccharide structures. Removal of carbohydrate chains by glycosylase enzymes results in a reduction of CD4 binding capacity.

Gruters (1987) reported that non-glycosylated and enzymatically deglycosylated gp120 could not inhibit cell fusion. Three inhibitors were examined for their effect

on sugar side chains. DMJ had no effect, as expected from a mannosidase inhibitor. However, the glucosidase inhibitors (DNJ and castanospermine) altered the glycosylase linked glucans to leave only glucose residues. After virus-producing cells had been grown for 72 hours in the presence of inhibitors, newly synthesised gp120 molecules were aberrantly glycosylated. syncytium formation was inhibited for 6 hours by DNJ and greatly reduced for several days. Castanospermine completely inhibited syncytium formation for several days. Inhibition of gp160 was also examined. It was concluded that the glucan structure on gp120 was responsible for syncytium formation. Even in the presence of inhibitors gp120 was produced in normal amounts by the virus. It was the presence of glucose residues and N-linked glycans that inhibited CD4 binding. The effect of DNJ was further examined by Shimizu et al. (1990). The effect of N-(3-phenyl-2-propenyl)-1-Deoxynojirimycin on the lectins binding to the HIV-1 glycoproteins was examined using biotinylated lectins of various sugar specificities as probes. The compound inhibited HIV-1 induced syncytium formation and viral infectivity of HIV-1 without cytotoxicity. In a study of the glycosidase pathway utilised between the viral and cell membrane glycoproteins. Montefiori et al. (1988) reported that different glycosidase enzymes in the endoplasmic reticulum are inhibited by different inhibitors. Castanospermine and DNJ specifically inhibit glucosidase I. Bromocondritolin inhibits glucosidase II,

1-deoxymannojirimycin inhibits mannosidase I and finally swainsonine inhibits mannosidase II.

1.10.3 The synthesis of DNJ

There are now completely synthetic systems to make DNJ and its precursor NOJ. Zeigler et al. (1988) described the synthesis of DNJ and DMJ from L-(+)-tartaric acid. Another raw material was used by Dax. et al. (1990) when DNJ was synthesised by a simple sequence of 6 or 7 steps from D-glucofuranurono -6-3-lactone. Lactone in the same form was also used by Anzeveno and Creemer (1990) to synthesise DNJ and NOJ. The process involved stereo selective amination of 5 - hydroxy -1, 2-0-isopropylidene α -D- xylo - hexofuranurono -6, 3-lactone into the respective products. Alternatively, Pederson et al. (1988) prepared DNJ and DMJ from glucose, via a catalysed condensation reaction by fructose diphosphate aldolase. DNJ and DMJ were produced in a ratio of 1:4 respectively by reductive amination along distinct reaction paths. A similar synthetic process for DNJ production was proposed by Reitz and Baxter (1990). This method involved a double reductive amination of commercially available 5-keto-D-fructose. The flexibility of the chemistry of these compounds was demonstrated by Glaser and Perlin (1988). The activity of the DNJ molecule against α -D-glucosidases was enhanced by attachment of methyl groups to DNJ. Schueller and Heiker, (1990) took DNJ and synthesised 2-acetamido -1,

2- dideoxy - galacto - nojirimycin.

The alternative method of synthesising DNJ was developed by Kinast and Schedel (1981). A combined chemical and microbiological process converted 1- deoxy - 1 amino-sorbitol by catalytic hydrogenation to DNJ.

DNJ can also be estimated in broths of *S.lavendulae* by using trehalase as a screen (Murao and Miyata, 1980). The DNJ was extracted by two Dowex ion exchange columns and a yield of 350mg from 12 litres was obtained. Ezure et al., (1985) patented *S.lavendulae* as a producer of DNJ in 1984. However, it is also possible to obtain DNJ from fermentation broths of *Bacillus* (Schmidt et al., 1979) and (Puls et al., 1980).

One of the most comprehensive studies on the biosynthetic pathway of DNJ and its related compounds was conducted by Hardick et al. (1992), using *S.subbrutilus* a member of the same taxonomic group as *S.lavendulae*. Deuterated glucose was used as a precursor and NMR spectroscopic analysis of the peracetylated products (DMJ and DNJ) showed glucose to be the precursor of NOJ, MOJ, DNJ and DMJ. The biosynthetic pathway illustrated in Fig 1.6 allows an estimation of the number of enzymatic steps involved in the synthesis of DNJ.

Fig 1.6

THE BIOSYNTHETIC PATHWAY OF DEOXYNOJIRIMYCIN BIOSYNTHESIS

An estimated 7 or 8 enzyme reactions (depending if fructose is oxidised or not) are required for the biosynthesis of deoxynojirimycin. The final reduction step involving the loss of oxygen may be chemically spontaneous, not involving a reductase enzyme.

DEOXYNOJIRIMYCIN BIOSYNTHESIS

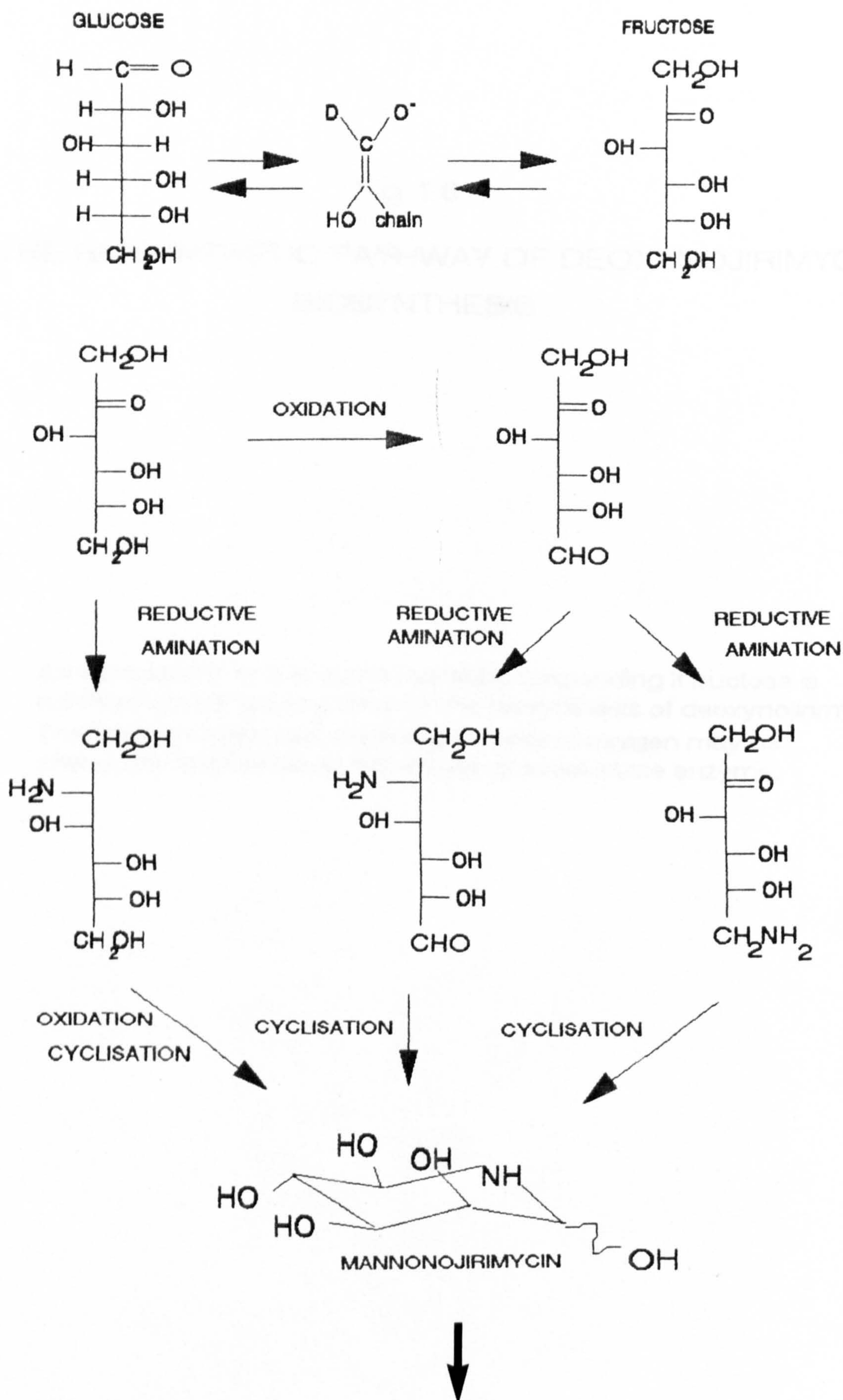
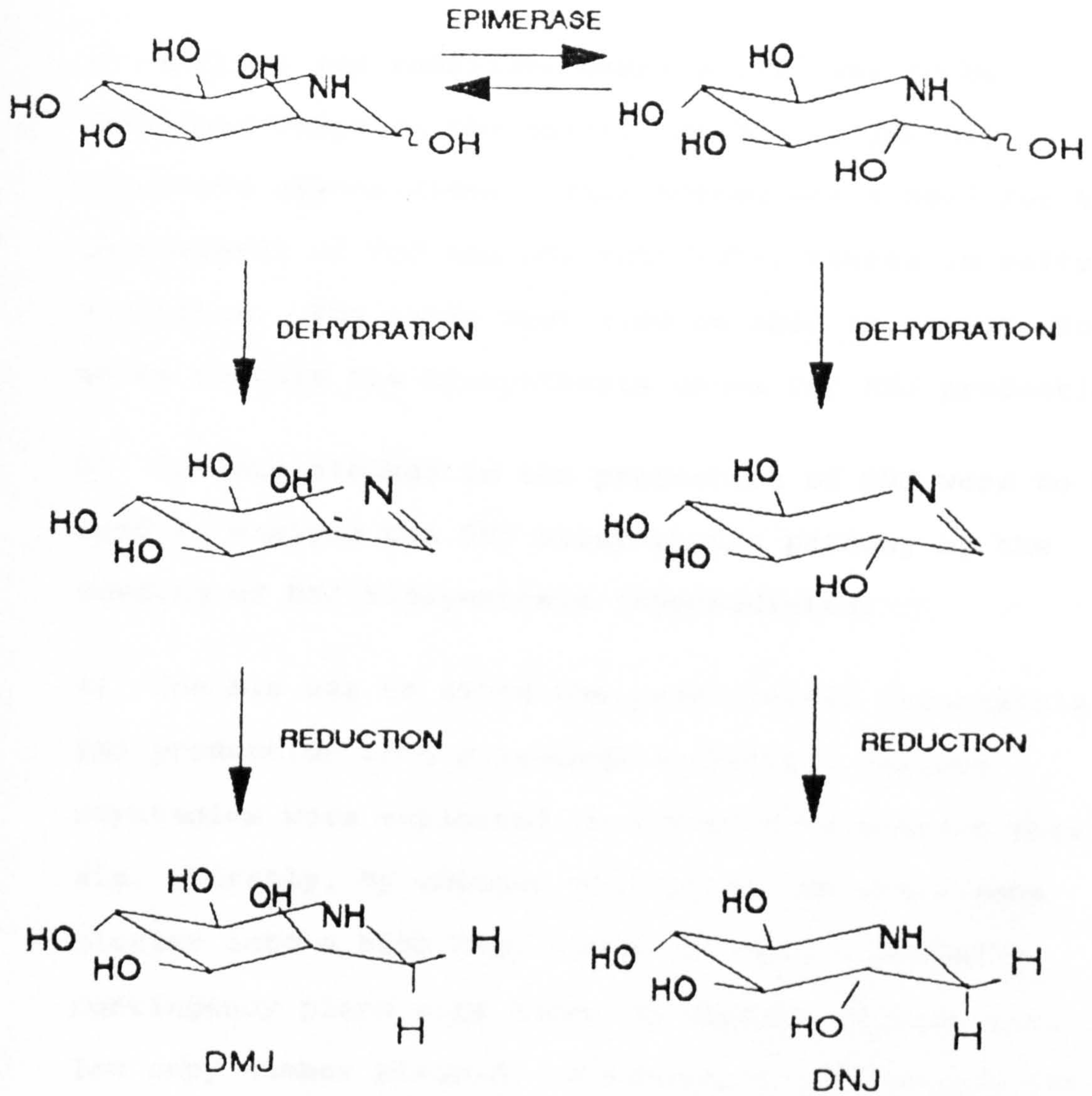


Fig 1.6 (cont'd)

CONVERSION OF MANNONOJIRIMYCIN TO DMJ AND DNJ



Taken from D.J. Hardick et al 1992.

Aims Of The Thesis

- 1) Physiological studies on the production of DNJ by selected members of the *S.lavendulae* species group were undertaken.
- 2) A quick and sensitive assay screen was to be developed based on the ability of DNJ to inhibit selective glycosidases. This screen was a tool for the measurement of NOJ and DNJ antibiotic titres in culture filtrates. The assay must also be able to detect clones which contain the biosynthesis genes for DNJ production.
- 3) Mutants blocked in the production of DNJ were to be used to analyse the DNJ biosynthetic pathway by the feeding of DNJ biosynthesis intermediates.
- 4) The aim was to clone the gene cluster responsible for DNJ production from *S.lavendulae* 31434. Various strategies were exploited in attempts to achieve this aim. Firstly, by shotgun cloning of the whole gene cluster into a high copy number plasmid. Secondly, contingency plans were based on shotgun cloning into a low copy number plasmid. A library would be made and the target gene selected for by complementation of mutants blocked in the production of DNJ.

CHAPTER TWO

MATERIALS AND METHODS

2.1 The *S.lavendulae* species group.

The eleven members of this group are equivalent to the taxonomic cluster 61 as defined by Williams et al. (1983). This work focused on three members of the group, *S.lavendulae* 5069, *S.subbrutilus* and *S.racemochromogenes*, as well as *S.lavendulae* 31434 which was later designated as being a member of this group as a result of forty one morphological or physiological tests. The full membership of the *S.lavendulae* species group is illustrated in Table 2.1.

2.2 Spore suspensions

Frozen spore suspensions of *S.lavendulae*, *S.subbrutilus*, *S.racemochromogens* and *S.lavendulae* 31434 were thawed and subcultured onto oatmeal agar (appendix 1). After sporulation the culture was harvested into 20% (v/v) glycerol and filtered through cotton wool to separate the spores from mycelium. Spore numbers were determined using a haemocytometer and spores were stored at -20°C in 2ml volumes until required.

Table 2.1

**THE SPECIES COMPOSITION OF THE S.lavendulae
SPECIES GROUP.**

Accession (Strain) number	Strain name
5558	<u>S.colombiensis</u>
5190	<u>S.goshikiensis</u>
5069	<u>S.lavendulae</u>
5445	<u>S.subrutilus</u>
5152	<u>S.flavotricini</u>
5178	<u>S.toxytricini</u>
5550	<u>S.Katrae</u>
5316	<u>S.polychromogenes</u>
5194	<u>S.racemochromogenes</u>
5216	<u>S.lavendulocolor</u>
5175	<u>S.roseviridus</u>

2.3 Shake flask experiments

Culture broth (30mls) was placed into Erlenmeyer flasks (250ml). The media used for growth and assays of DNJ production were: malic acid, minimal glucose, ISP4 (appendix 1), glycerol proline and a recommended DNJ medium (appendix 1). The flasks were sterilised, inoculated with 100 μ l of spore suspension and incubated at 28°C on a Gallenkamp orbital shaker at 250rpm.

2.4 The trehalase enzyme assay

The bioassay used 3 eppendorf tubes for each sample assayed. These were a blank (containing no enzyme), a positive control (no supernatant) and the actual test sample. Trehalase enzyme, 100 μ l (0.2 units) (Sigma) was made up to 10mls with 0.1M maleate buffer pH 6.0. Trehalose substrate (56mM) was made by dissolving 210mg of trehalose in 10mls of 0.1M maleate buffer.

	Blank	100%	Test
Distilled Water	20 μ l	20 μ l	0
DNJ or Supernatant	20 μ l	0	20 μ l
Enzyme	0	20 μ l	20 μ l

These samples were incubated at 37°C for fifteen minutes before 20µl of trehalose substrate was added to each tube and incubated at 37°C for one hour. Glucose GOD-PAP Diagnostic Kit solution (1ml) (Boehringer Mannheim) was added to each tube; followed by 20µl supernatant to the 100% eppendorf (to ensure all tubes contain equal volumes of supernatant). Distilled water (20µl) was added to the other tubes to equalise volumes before being incubated at 37°C for 30 minutes. The absorbance was read at 510nm against the blank sample.

$$\frac{\text{Absorbance } 100\%}{\text{Absorbance of Sample}} \times 100\% = \text{Percentage inhibition due to supernatant.}$$

The DNJ concentration was derived from a standard curve (appendix 1 Fig 7.1).

2.4.1 DNJ production on solid media

Several solid media were examined for growth: MGA, DNJ Basal Medium, Glycerol proline medium, ISP4 and ISP5 (Appendix 1). The aims of this experiment were not only to determine the optimal medium, but also the onset of DNJ production and suitable times to assay for DNJ.

Spore suspensions (15µl) were inoculated onto the solid media plates. Each day a piece of agar 10mm in diameter was cut out around the colonies. It was suspended in 1ml of distilled water, dried in a rotary

evaporator, and taken up in 50 μ l of distilled water 20 μ l of this sample was assayed for DNJ using the trehalase assay.

2.5 Conversion of nojirimycin fed as bisulphite adduct, to nojirimycin.

A small amount of non-absorbent cotton wool was placed inside a Pasteur pipette at the narrow end, supporting a column of Dowex Ix8 ion exchange resin, which half filled the pipette. The resin was washed by adding 10ml of 1M NaOH, followed by 10ml of water. Converted resin (1ml) was added to 10ml of nojirimycin bisulphite suspension. When the resin was added the nojirimycin bisulphite dissolves. The NOJ bisulphite solution was run through the column before an additional 10ml of distilled water was added to the top of the column. A solution containing NOJ (18ml) was collected and freeze dried over 24h to harvest the nojirimycin. The white crystalline powder was taken up in 5ml of sterile distilled water.

The nojirimycin solution was centrifuged for 10 minutes at 13400g to remove any undissolved nojirimycin bisulphite. The concentration of the NOJ in the supernatant was quantified using the α -glucosidase assay system. The concentration of nojirimycin in the solution was estimated to be 9×10^{-7} M.

2.5.1 Feeding blocked mutants with antibiotic biosynthesis intermediates.

The 5ml of nojirimycin solution available confined the experiment to 2 (50ml) shake flasks. DNJ basal medium was made up and 5ml was added to each of the flasks. A spring was inserted into each flask to break up the mycelium. The flasks were sterilised and then inoculated with 40 μ l spore suspension of the mutant, blocked in the production of both NOJ and DNJ. The flasks were incubated at 28°C and shaken at 200rpm in an orbital shaker.

After 24h, 5ml of NOJ solution was filter sterilised and added to one of the flasks. The volume of the other flask was equilibrated by adding 5ml of sterile distilled water.

Each day, for seven consecutive days, 300 μ l of broth was extracted and centrifuged at 3352g to remove all components of the medium. The supernatants were then analysed for DNJ and NOJ by testing the supernatants with the α -glucosidase assay system before and after boiling in 6M HCL for six hours.

2.6 Genetic Manipulation Of *Streptomyces*

2.6.1 Total chromosomal DNA isolation

Cultures of Tryptone Soya Broth (50ml) were inoculated with *S.subbrutilus* and *S.lavendulae* (31434). The Erlenmeyer flasks were inoculated with 100µl of spore suspension (10^8 /ml) incubated at 30°C, whilst shaken in an orbital shaker at 150-200rpm. Mycelium (50mg) was resuspended in 500µl of lysozyme solution and incubated at 37°C for 30min until the cells were translucent. SDS 2%w/v (250µl) was added and vortex mixed. 250µl of neutral phenol-chloroform solution was added, vortexed and spun in a microcentrifuge for two minutes. The supernatant was removed, leaving only the white interface. This was repeated until no interface was visible. Sodium acetate, (3M) 0.1 vol, was added, then one volume of isopropanol before mixing and leaving at room temperature for 2 min. This solution was centrifuged at 13900g for 30sec and all the liquid was removed. The pellet was redissolved in 500µl TE and stored at 4°C.

2.6.2 Quantification of DNA

DNA solution (10µl) was diluted in 990µl of TE (Tris EDTA) buffer (appendix 2).. Spectrophotometer readings were taken at 260nm and 280nm after being blanked against

TE. An optical density of 1 is equivalent to 50 μ g/ml for double stranded DNA. The purity of the DNA was checked by running a small sample (1 μ l) on an agarose gel.

2.6.3 Restriction digest of DNA

DNA from each host strain (30 μ l at a concentration 1 μ g/ml) was placed in a separate eppendorf. Then 6 μ l of buffer (Amersham, supplied with restriction enzyme) was added, along with 2 μ l RNase (20 μ g/ml). The solution was made up to a final volume of 60 μ l with sterile distilled water. 2 μ l (15 units) of the restriction enzyme Sau 3A was added to each sample before being incubated at 37°C. At two time intervals 30 and 45 seconds, 20 μ l was taken and analysed by gel electrophoresis.

2.6.4 Size fractionation using agarose gel electrophoresis

The concentration of agarose used was 0.8% to give better separation of fragments. The buffer used was Tris-borate buffer (appendix 2) and the gel was run for 45min at 50v.

2.6.5 Recovery of DNA fragments from agarose gels

The 1 kb Ladder (BRL) was used as a guide for the excision of DNA. Fragments >12 Kb up to the origin at the top of the gel were removed and placed in the wells of the Electro eluter. This was flooded with TBE (appendix 2). The troughs of the Electro eluter were filled with 7.5M potassium acetate coloured with bromophenol blue. Elution was carried out at 100V for 30 minutes. Potassium acetate was removed from the troughs and added to two volumes of 100% ice cold ethanol. The DNA was left to precipitate overnight at -20°C. DNA was re-extracted as in 2.6.1.

2.6.6 Protoplast formation and enumeration

Frozen spore suspensions of *S.lividans* (TK24) were thawed and subcultured onto R5 (appendix 2) which had been diluted 100% with water. When the culture had sporulated the spores were harvested and stored at -20°C in 20% (v/v) glycerol. The number of spores was determined using a haemocytometer.

Protoplast formation

After sterilisation, 30mls of Yeast Extract-Malt Extract Medium (YEME, Appendix 2) was inoculated with 75 μ l (10^8 /ml) of spore suspension. This was incubated for 36-

40h at 28°C in a orbital shaker. The remaining methodology is exactly as in Hopwood et al. (1985).

Enumeration

The concentration of protoplasts was estimated using the haemocytometer and adjusted to approximately 10^9 /ml by dilution or centrifugation. The protoplasts were stored in 50 μ l aliquots of protoplast buffer (appendix 2), in eppendorfs which were placed in ice and frozen slowly to -70°C. They were thawed rapidly when required.

2.7 Plasmid preparation

A spore suspension of *S.lividans* (200 μ l) was inoculated into 500ml TSB containing 50 μ g/ml thiostrepton. A spring was placed inside the flask to hinder the formation of mycelial pellets and improve aeration.. The flask was shaken at 200rpm on an orbital shaker for 3 days at 28°C. The culture was dispensed into 250ml centrifuge bottles (two for each flask) and centrifuged at 11307g for 10 minutes. The mycelium was washed with 10% sucrose (w/v) and centrifuged again at 11307g for 10 minutes. The pellet was resuspended in 45ml of 25mM Tris-Hcl, 25mM EDTA, 0.3M sucrose pH8. Lysozyme powder (10mg) was added and the solution was incubated at 37°C for at least 30 minutes, with occasional shaking. The cells were lysed with 30ml of 0.3M NaOH, 2% SDS (w/v) which was made up fresh, added to each sample, mixed well and incubated at

70°C for 15 minutes. The samples were left to cool to room temperature. Acid phenol-chloroform (20ml) (appendix 2) was added, mixed very thoroughly, and centrifuged at 13900g for 15 minutes at room temperature. The upper aqueous phase was decanted into a new bottle and 7ml of unbuffered 3M sodium acetate and 70ml of isopropanol were added and the samples were left for 20-30 minutes to precipitate. The precipitate was brought down by centrifugation at 13900g for 20 minutes. The supernatant was discarded and dissolved in 10ml TE buffer (appendix 2) containing 50mM NaCL. 50 μ l of RNase (50 μ l at 10mg/ml in 0.3M sodium acetate pH 4.8, pre-heated to 90°C for 10 minutes to inactivate DNases) was added and left overnight in the TE. Sodium acetate 3M, (1ml) and 5ml of neutral phenol-chloroform were added and the samples were shaken vigorously. The samples were centrifuged for 10 minutes at 4°C and the upper aqueous phase was decanted into another bottle. Chloroform (5ml) was added to this aqueous phase, shaken, and centrifuged at 3438g for 2 minutes. Again the upper aqueous phase was removed and the chloroform extraction was repeated. Isopropanol (10ml) was added to the resultant aqueous phase and mixed. The samples were left for 30 minutes to precipitate and centrifuged at 13900g for 20 minutes. The pellet was washed in 1ml of absolute etOH and then dried under a vacuum. Caesium chloride gradients were performed in 5ml Quick-seal tubes according to the standard procedure given in Hopwood et al. (1985). Plasmid DNA was removed from the tubes by hypodermic

needle under long wave (300-360nm) illumination. The ethidium bromide was extracted by adding an equal volume of 1-butanol saturated with aqueous 5M NaCl in TE buffer. The phases were mixed, allowed to separate and the upper phase was discarded. This process was repeated until the pink colour disappeared from the upper phase. The DNA was extracted from the aqueous phase by ethanol precipitation described in Hopwood et al. (1985). The prepared plasmid 5µg was then linearised by the addition of 1µg of BglII in a volume of 54µl of distilled water and 6µl of restriction enzyme buffer. The samples were incubated for three hours at 37°C. The plasmid was phosphatased by the addition of 1µl (1 unit) of alkaline phosphatase. The samples were again incubated at 37°C for three hours. The action the phosphatase enzyme was terminated with the addition of 40µl phenol-chloroform. After being centrifuged for 10mins at 3438g. The upper phase was removed and 50µl of chloroform was added. Isopropanol (10µl) was added and the samples were left 30mins to precipitate, before being centrifuged at 13900g for 20mins. The pellet was washed in 500µl of absolute alcohol and dried under a vacuum. The plasmid was dissolved in 20µl TE and stored at 4°C.

2.7.1 Ligation Conditions

The ligation mixture consisted of 4µl of DNA fragments (2µg) either from *S.subtrutilus* or *S.lavendulae* added to 0.5µl of plasmid DNA (0.5µg), either pIJ940, pIJ916 or pIJ702. 1µl of ligation buffer (appendix 2) was added,

together with 1 μ l of T4 DNA ligase (BRL). The whole volume was made up to 10 μ l with 3.5 μ l of sterile distilled water. The mixture was incubated overnight at 15°C. The product was then stored at 4°C until utilised in transformations. The ligation was observed by taking 1 μ l of the completed ligation mix. This was added to 1 μ l loading dye (containing bromophenol blue), made up to a volume of 5 μ l by adding 3 μ l of water and run on a 1% agarose gel for 40 minutes at 50V.

2.7.2 Transformation conditions

The methodology for this was based on the rapid transformation method of Hopwood et al. (1985). T (transformation) buffer (appendix 2) was preferred to P buffer and the final concentration of thiostrepton was reduced from 50 μ g/ml to 35 μ g/ml. Transformants grew through the overlay after 2 days and were picked off after 5 days.

TSB medium containing 25 μ g/ml thiostrepton was dispensed in 200 μ l aliquots, into 96 well microtitre dishes, by a Titertek Multidrop. One transformant colony was transferred to each microtitre well. The colonies were incubated at 27°C in a sealed plastic bag, to maintain humidity and prevent desiccation. They were then transferred to another microtitre plate by using a 96 well inoculator every 10 days.

2.7.3 Plasmid "Minipreps"

Transformants were picked off and grown in 250ml shake

flasks containing 30mls of TSB and 25µg/ml thiostrepton. The flasks were incubated at 28°C and shaken at 200rpm on a Gallenkamp orbital shaker, for 3 days. The "miniprep" method is identical to that of Hopwood et al. (1985). The only modification was an extra purification step using neutral phenol. After the DNA pellet had dissolved in 50µl T.E., 150µl of sterile distilled water was added followed by 80µl of neutral phenol. After centrifugation (13400g for 5 minutes) the upper phase was removed and the remaining phenol was extracted by adding 50µl of chloroform containing 4% iso amyl alcohol (v/v). After centrifugation of 13400g for 5 minutes the upper phase was removed and a 0.1 volume (18µl) of 3M sodium acetate was added. The DNA precipitated in the presence of 2 volumes of 100% absolute alcohol, overnight at -20°C. The centrifugation, washing and drying of the DNA was achieved using the standard protocol as described in Hopwood et al. (1985).

2.7.4 Characterisation of frequency and size of insert in recombinant plasmids.

The insert size was determined by cutting the plasmid with restriction enzymes. Usually *Bcl* I (Pharmacia) was added but *Eco*RI (BRL), *Bam*HI (BRL) and *Bgl*II (BRL) were also used. The prepared plasmid (1µg) was added to an eppendorf, with 7µl of sterile water, 1µl of 10 times buffer (supplied with enzyme) and 1µl of restriction enzyme. The enzyme was added in two 0.5µl aliquots, the

second aliquot, after 1 hour. The *EcoRI* and *BamHI* digests was incubated at 37°C and the *BclI* digests at 50°C, for 3 hours.

To view the digests on a gel, 6µl of water and 2µl of loading dye were added to each digest. This was run on a 0.8% agarose gel overnight at 20V.

The location of insert was calculated by comparing the digests of recombinant plasmids with digests of the wild type. The size was determined by comparisons with the *HindIII* marker. This data was used to plot the logarithm of lambda DNA against the distance moved on a gel. This allows the size of other restricted plasmid bands to be determined.

2.7.5 Transformation frequency

This was estimated from the ligation photographs. The intensity of the ligation band that contained the insert was compared to the intensity of bands in the 1Kb ladder or *HindIII* marker. The amount of DNA contained in the marker was an indication of the quantity of DNA that the ligation contained to produce the transformants.

2.7.6 Transformations using the modification mutant ZX1.

Spores of the *S.lividans* ZX1 mutant were kindly donated by Dr. M.J. Buttner from the John Innes Institute. These were plated (50µl) onto the recommended ZX1 agar medium (appendix 1). After seven days incubation at 30°C the

spores were harvested into 20% (v/v) glycerol and stored at -20°C until required.

S.lividans protoplasts were made by the standard method in Hopwood et al. (1985). The protoplasts were transformed with uncleaved vectors pIJ702 and pIJ916 and recombinant vectors pIJ702 and pIJ916, containing DNA fragments of *S.lavendulae* and *S.subbrutilus* respectively.

2.8 Probing with the Digoxigenin Non-radioactive DNA labelling and detection kit.

2.8.1 Non-Radioactive labelling of DNA

Approximately 2µg of pIJ940 DNA was digested with *Bgl*III in a volume of 10µl. Once the digest was complete 2µl were taken and made up to a volume of 15µl with water. This was denatured at 95°C for 10 minutes and then placed immediately on ice. Hexanucleotide mixture (2µl) was added together with 2µl of dNTP Labelling mix. Addition of 1µl of Klenow enzyme made the total volume up to 20µl which was incubated overnight at 37°C. To halt the reaction 2µl of 0.2M EDTA pH8 was added, followed by 2µl of lithium chloride (10M) and 60µl of ice cold ethanol. This was kept at -70°C for one hour before the total volume was centrifuged at 13400g for ten minutes. Ice cold 70% ethanol (100µl) was added to wash the DNA. The sample was then dried in a vacuum dryer. The DNA was dissolved in 50µl of TE, 5µl of this solution was run on a 0.8% agarose gel and the remaining 45µl was divided into 3 aliquots and frozen at -20°C until required.

2.8.2 Southern hybridisation

Agarose gels were soaked twice for ten minutes in 0.25M HCl with gentle rocking. After rinsing in water the DNA was denatured by soaking the gel twice in 0.5M NaOH, 1M NaCl for 15 minutes. This was rinsed three times in water. The gel was neutralised by soaking in 3M NaCl, 0.5M Tris pH7.5 for twenty minutes. The hybridisation transfer membrane (Hybond-N), was cut to size before being soaked in distilled water and then 2 X SSC. A Stratagene Posiblitter was used to blot the membrane. The reservoir sponge was soaked with 20 X SSC and run at a pressure of 85 to 90 mm of Hg. The posiblitter was left to run for 2 hours before the membrane was dried and the DNA was fixed to it by 3 minutes of UV radiation. The membrane was then sealed in a plastic bag prior to hybridisation.

2.8.3 Hybridisation of the probe to target DNA

The membrane was immersed in 100mls of hybridisation solution (appendix 2) and rocked gently overnight at 70°C. The following day the membrane filter was placed between two pieces of Hybaid nylon mesh before being put into a Hybaid glass tube (HB-OV-BL). Fresh hybridisation solution was made up and 4mls was added to the tube.

Concomitantly 0.6 μ g (a 15 μ l aliquot) of probe was denatured at 95°C for 10 minutes and put immediately on ice. Hybridisation solution (1ml) was added to the probe

before being put into the glass tube with the membrane filter. The tube was left overnight in a Hybaid mini hybridisation oven, which had been preheated to 70°C.

2.8.4 Development of blots

The membrane, still inside the Hybaid tube, was washed twice with at least 50mls of 2 X SSC, 0.1% SDS (w/v) at room temperature. The membrane was then washed twice with 0.1 X SSC 0.1% SDS, for 15 minutes at 70°C. The membrane was removed from the Hybaid tube and washed for 1 minute in tris/NaCl buffer (buffer 1 appendix 2) at room temperature. Then incubated for 30 minutes with 100mls of blocking agent buffer (buffer 2 appendix 2) at room temperature. Again the membrane was washed with buffer 1 before incubation for 30 minutes with 40mls of buffer 1 containing 150mU/ml antibody conjugate. Two successive washes with 100mls of buffer 1, for 15 minutes, preceded a final equilibrating of the membrane by 40mls of tris/NaCl/MgCl₂ (buffer 3 appendix 2) for 2 minutes. The membrane was immersed in 40mls of freshly prepared colour solution (appendix 2) and allowed to fully develop in the dark. The reaction was terminated after 3 hours by washing the filter in 50mls of TE. After drying upon filter paper the membrane was placed inside a polythene bag.

2.9. Preparation of phage

The initial preparation of a high titre KC301 phage preparation was obtained using the standard method in Hopwood et al., (1985). Two different lysates of KC301 were produced, one through infection and replication in *S.lividans* and the other through replication in *S.lavendulae*. The lysates were quantified by diluting 100 μ l in a tenfold series with nutrient broth (Difco). Nutrient agar plates containing 0.5% glucose and the appropriate divalent cations had already been prepared. 100 μ l of the lysate and lysate serial dilutions down to 10⁻⁵ from *S.lividans* were dispensed onto the 5cm dishes of Nutrient agar in duplicate. This procedure was repeated with lysate from *S.lavendulae*. Soft nutrient agar (800 μ l, 3g/l) containing spores of the indicator strain (either *S.lividans* or *S.lavendulae*) was added and swirled to cover the whole plate. Once set, the plates were incubated overnight at 30°C. The plaques were scored to indicate the number of phage per ml for the two lysate solutions. Each of the lysates were used to infect both *S.lividans* and *S.lavendulae*, to examine if any restriction system exists between the two strains.

2.10 Assay of α -glucosidase inhibition

The enzyme used in the assay was α -glucosidase (Sigma). The enzyme (100units) was taken up in 1000 μ l phosphate buffer pH 6.8. (One unit liberated 0.18mg of glucose at 37°C at pH 6.8). The substrate was p-nitrophenyl α -D-glucopyranoside and a working concentration of 3mg/ml was made up in water and used as a stock solution.

2.10.1 Optimising the absorption wavelength

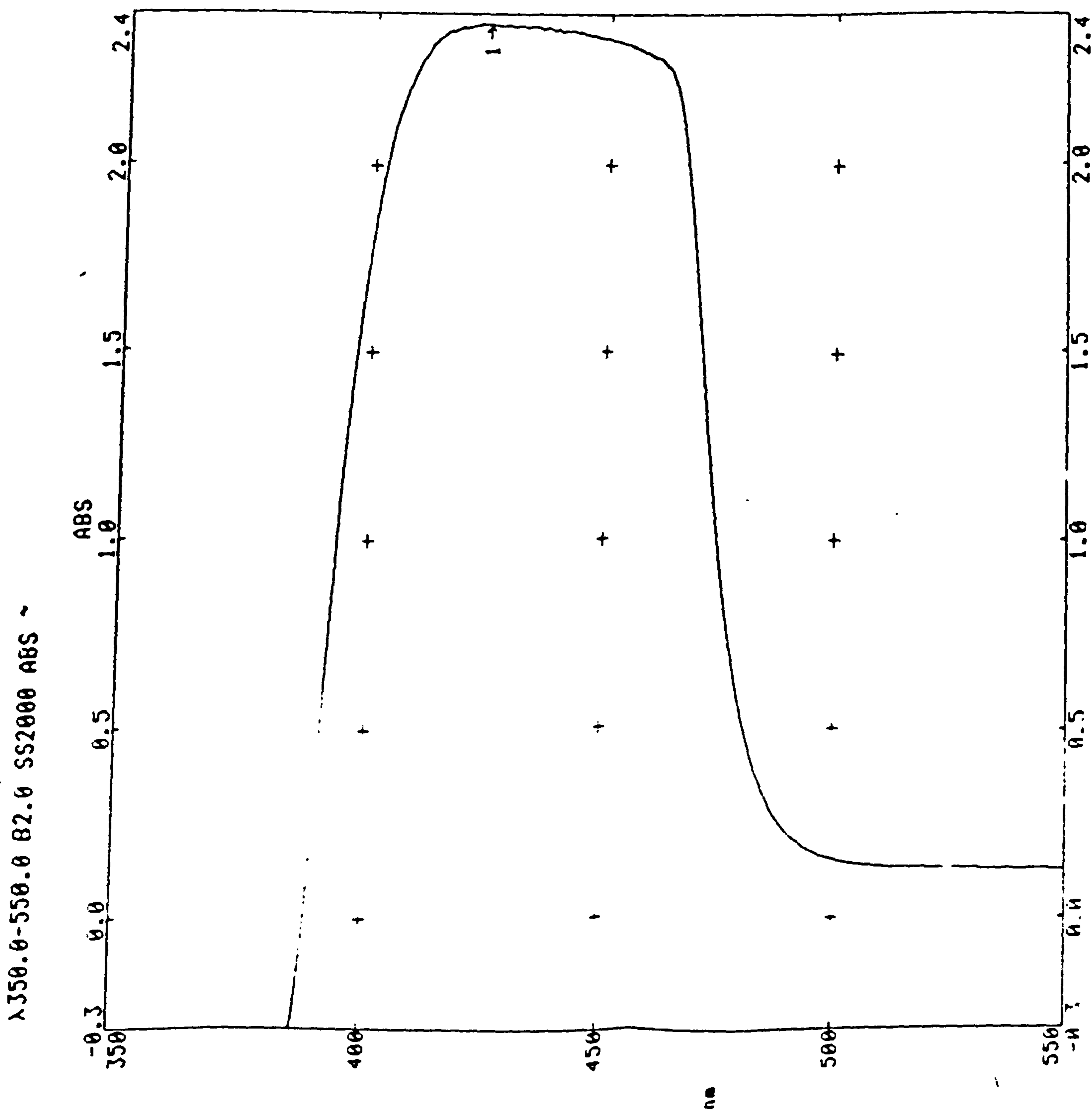
Glucopyranoside solution 810 μ l at 3mg/ml was added to a quartz cuvette and used to blank the spectrophotometer. The reaction cuvette contained 800 μ l of substrate and 10 μ l of glucosidase enzyme (1 unit). The reaction temperature was 37°C and ran for 45 minutes. This was scanned between 350nm and 550nm, to determine the optimal wavelength, to scan the p-nitrophenol absorption. An control containing no enzyme was used to blank the spectrophotometer Fig (2.1).

2.10.2 The optimal amount of substrate

The final substrate concentration of α -D-glucopyranoside substrate ranged from 9.2×10^{-5} M. to 1.15×10^{-2} M. in a volume of 200 μ l and was added to 8 μ l of glutathione (1mg/ml) purchased from Sigma and 0.1 units of enzyme added in 10 μ l of phosphate buffer.

Fig 2.1

Absorption Spectrum For p-Nitrophenol liberated from glucopyranoside by α -glucosidase



These quantities were added to 12 wells of a 96 well microtitre plate. Absorbance readings were taken using the Titertek Multiscan MK II platereader. Table 2.2 below shows the volume of assay solutions in each of the microtitre wells assayed. The control utilised in the assay procedure, was a control to check against natural colouring caused by p-nitrophenyl.

2.10.3 The optimal amount of enzyme

The enzyme concentrations of 0.5, 0.1 and 0.025 of a unit were stored as frozen aliquots in 1000 μ l phosphate buffer. The enzyme concentrations were examined in turn to determine a concentration at which all the enzyme was used to make product.

2.10.4 An experiment to determine the inhibitory effect of deoxynojirimycin.

Deoxynojirimycin (DNJ) was purchased from Sigma and dissolved in water at the following range of concentrations: 0.02, 0.01, 0.005, 0.001 and 0.0005mg/ml. Table 2.3 shows the amount of enzyme, substrate and inhibitor added to each microtitre well.

This layout was repeated for each concentration of DNJ.

Table 2.2

DEVELOPMENT OF THE α -GLUCOSIDASE ASSAY

	Subtrate conc	Subtrate conc	Subtrate conc	Subtrate conc	Enzyme
	9.2×10^{-5} M	4.6×10^{-5} M	2.3×10^{-5} M	1.15×10^{-5} M	blank
Substrate volume	20	10	5	2.5	10
Glutothione	8	8	8	8	8
Enzyme volume	10	10	10	10	0
Water volume	172	182	187	189	192

Values in μ l

Table 2.3

THE INHIBITION OF α -GLUCOSIDASE BY DEOXYNIJIRIMYCIN AND NOJIRIMYCIN

	Substrate conc	Substrate conc	Substrate conc	Substrate conc
	9.2×10^{-5} M	4.6×10^{-5} M	2.3×10^{-5} M	1.15×10^{-5} M
Substrate volume	20	10	5	2.5
Glutathione	8	8	8	8
Enzyme volume	10	10	10	10
Inhibitor volume	22	22	22	22
Water volume	150	160	165	167

Values in μ l

Experiment to determine the detection limits of the assay.

This involved repeating the assay with the concentration of DNJ 2.5×10^{-7} M. (0.0005mg/ml) that gave no inhibition.

2.10.5 Experiment to determine the inhibitory effect of nojirimycin.

Nojirimycin (NOJ) was kindly provided from the Department of Chemistry, Warwick University. It was added to the assay in quantities and concentrations identical to those used for DNJ.

2.10.6 Inhibition produced from experimental media

Two media were tested in the assay. ISP4 and DNJ basal medium (appendix 1) were both made up fresh and 5mls were centrifuged at 3000rpm. The supernatants were tested independently in the assay by adding 22 μ l to the microtitre wells.

2.10.7 Production of DNJ from *Streptomyces* strains in two media.

DNJ basal medium and ISP4 medium were made up and 50mls was dispensed into 250ml Erlenmeyer baffled flasks. They were shaken on an orbital shaker at 200rpm and

incubated at 28°C. Two replicate flasks were inoculated for each of the 3 strains, *S.lavendulae* (31434) *S.subbrutilus*, *S.lividans* and a medium blank. Only the DNJ producing *S.lavendulae* (31434) strain was examined in the enzyme assay. Culture medium (1ml) was removed from the two flasks each day and was centrifuged at 5000rpm, to remove any suspended medium. The supernatant was dispensed in 22 μ l aliquots into the microtitre wells and tested in the α -glucosidase assay. The 100% water blank involved replacing the added supernatant with 22 μ l water. Any significant difference between the data was examined using the Genstat statistical package.

2.10.8 Determination of the detection limits for DNJ and nojirimycin using the α -glucosidase Assay.

Supernatants of *S.lavendulae* 31434 (50 μ l), which had been stored at -20°C, were made up to 500 μ l with sterile distilled water. The diluent was assayed in an identical manner as the original supernatant, by adding 22 μ l to the microtitre wells.

2.11 Microtitre plate screening of transformants

DNJ basal medium (210 μ l) was dispensed into each well of a 96 well membrane test plate using a Titertek Multi-pipette. The test plate (Pall biosupport Silent Monitor) had 0.45 micron loprodyne membrane-bottomed wells to allow filtering of the well contents. The culture broth

within these wells was inoculated from transformants grown in microtitre wells containing TSB agar and 25µg/ml thiostrepton. The transfer involved use of the Dynatech Inoculator MIC-2000 a multi-inoculator. In addition to plates of transformants, several plates served as controls including 72 wells of one plate which were inoculated with *S.lavendulae* as a positive control and two whole plates of *S.lividans*, as negative controls. Two plates each of *S.lividans* transformed with the plasmids pIJ702, pIJ940 and pIJ916 were examined to take into account the inhibitory effect of transformed *S.lividans*.

2.11.1 The primary screen

DNJ medium (210µl) was added to the wells of the microtitre plates. On each plate, *S.lavendulae* was added to one well, as a positive control and *S.lividans* was added to another well as a negative control. All the plates were incubated at 27°C, without shaking and after 8 days the plates were removed and the supernatants were filtered into another microtitre dish through the basal membrane. The filtrate (25µl) was dispensed into another microtitre dish which contained all the solutions necessary for the enzyme assay, except the enzyme itself. Addition of the enzyme initiated the reaction, while the actual screening process used the method previously described in (2.10.4).

2.11.2 Secondary screening

The 10% of transformants (140) that produced the most inhibition in the enzyme assay were re-grown and re-assayed by repeating the methodology of the primary screen.

2.11.3 Tertiary screening

The 15% of transformants (20) that produced the most inhibition in the enzyme assay, were picked off and inoculated into 30ml DNJ medium, in 250ml Erlenmeyer flasks. They were incubated at 27°C and rotated on an Gallenkamp orbital shaker at 200rpm for 8 days. The cultures were then centrifuged at 5000rpm and 22 μ l of the ultrafiltrated supernatants were assayed for inhibitors as previously described (2.10.4.).

CHAPTER THREE

A PHYSIOLOGICAL EXAMINATION OF THE *S.lavendulae* SPECIES GROUP AND THE CHARACTERISATION OF VARIOUS BLOCKED MUTANTS.

3.1 Introduction

S.lavendulae ATCC 31434 was patented as a producer of DNJ by Ezure et al. (1985). The eleven members of cluster 61 (the *S.lavendulae* species group) as outlined by Williams et al. (1983), were screened in this laboratory for glycosidase and amylase inhibitory activities (unpublished results). Further examination of these closely related strains for antibiotic production noted production of DNJ by *S.subbrutilus* ATCC 27467.

The work described in this chapter was to examine these two DNJ producers (*S.lavendulae* 31434 and *S.subbrutilus*) together with *S.lavendulae* ATCC 8664 and *S.racemochromogenes* ATCC 23954, two strains not known to produce DNJ. These four strains were tested for DNJ production on solid and in liquid culture. The other members of the *S.lavendulae* species group were not studied in detail for DNJ production using different growth systems. This information could be important for the development of an assay system to detect DNJ. It is well documented that *Streptomyces* species grow differently on a solid medium compared to broth culture. Substrate hyphae usually differentiate and sporulate on

solid media, while sporulation rarely occurs in liquid medium. More importantly, commercial production of antibiotics is concerned with liquid fermentations because high titres can be maximised in broth.

This study did not involve measurement of specific rates of antibiotic production but focused attention on the minimum titre of antibiotic that could be detected.

This Chapter also describes work done with three mutants produced by UV mutagenesis (Trew 1992). These were mutants of *S.lavendulae* blocked in the production of NOJ and DNJ. They did not complement each other but some precursor feeding experiments to confirm utilisation of biosynthetic intermediates were carried out and are described in this section.

3.2 RESULTS

3.2.1 DNJ production in broth cultures.

The differential production of DNJ in various media is illustrated in Table 3.1. Strain *S.lavendulae* 8664 did not produce DNJ in any of the media. *S.racechromogenes* was not a previously known producer of DNJ. However, a small amount of DNJ production was measured in the malic acid medium.

S.lavendulae and *S.subbrutilus* yielded the highest levels

TABLE 3.1

THE EFFECT OF GROWTH MEDIUM ON DNJ PRODUCTION
OF SELECTED *Streptomyces* STRAINS

The deoxynojirimycin was assayed after 96 hours by trehalase inhibition.

- No DNJ present.
- * DNJ present ($<10\mu\text{g/ml}$).
- * * DNJ present ($>10\mu\text{g/ml}$).

Legend for media in appendix

	MALIC ACID	MINIMAL GLUCOSE	ISP4	DNJ MEDIUM	GLYCEROL PROLINE
<u>S.lavendulae</u> 8664	-	-	-	-	-
<u>S.racemochromogenes</u>	*	-	-	-	-
<u>S.subbrutilus</u>	-	-	*	**	-
<u>S.lavendulae</u> 31434	*	*	**	**	-

of DNJ from DNJ basal medium. *S.lavendulae* 31434 showed the highest production titres and produced DNJ in four of the five media tested. Production by *S.subbrutilus* was limited to two of the five media and produced lower amounts of DNJ than *S.lavendulae*.

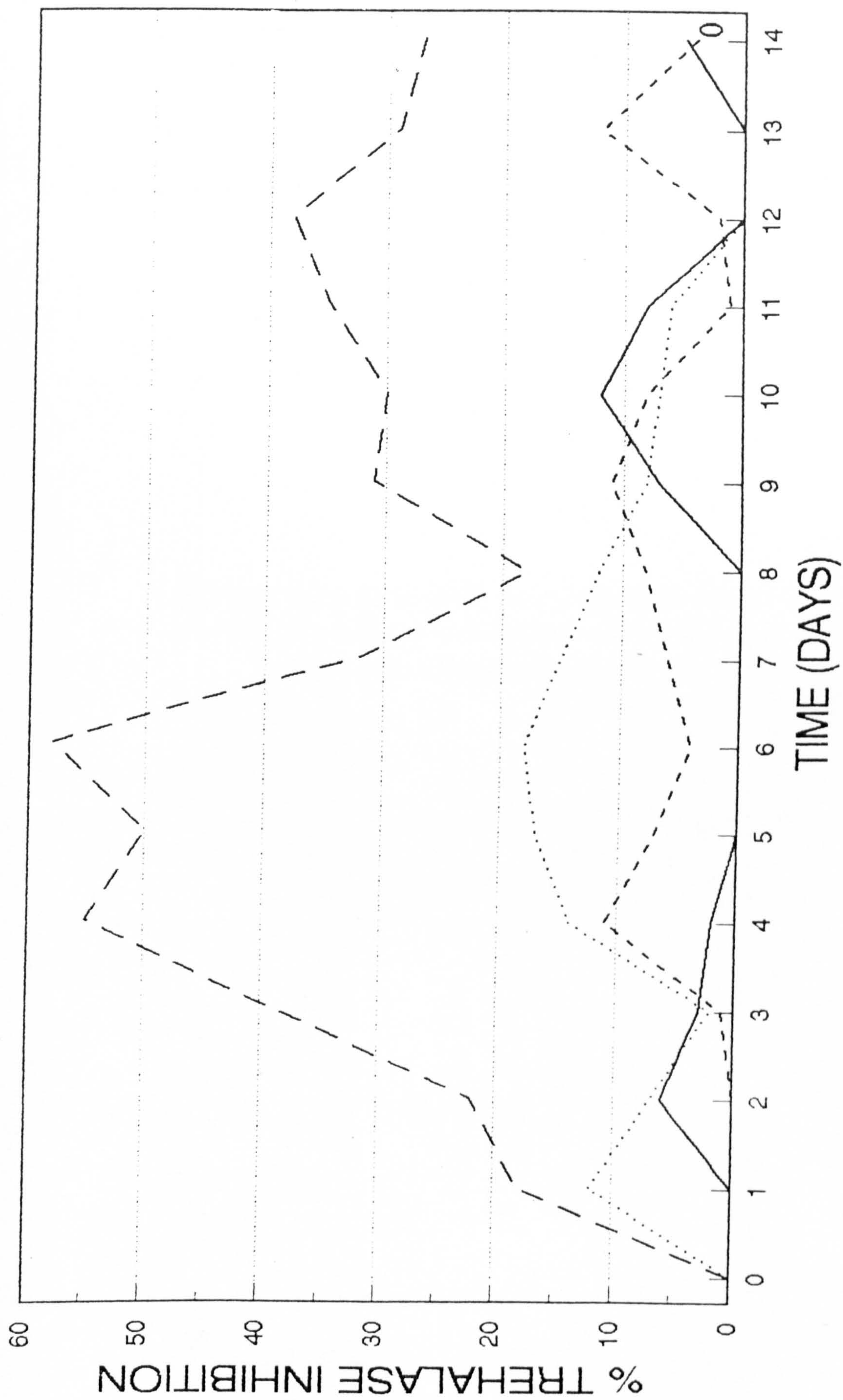
3.2.2 DNJ production on solid media

When growth and antibiotic production were examined on agar cultures, growth occurred only on the MGA and the DNJ basal medium agars, while DNJ production was only detected using MGA agar. The total amount of enzyme inhibition attributed to NOJ and DNJ production is shown in Fig 3.1. Trew (1992) estimated that DNJ inhibits the trehalase assay ten times more effectively than NOJ. Boiling in acid removed the NOJ and the absolute titre of DNJ is illustrated in Fig 3.2. There was no reduction of inhibition in the medium used as a control. *S.lavendulae* 8664 and *S.subbrutilus* both failed to produce any inhibition indicating these strains produced no DNJ or NOJ. The titre for *S.lavendulae* 31434 was 3×10^{-6} M. on day 4 and day 7. The same strain in liquid culture produced 4×10^{-5} M. (Fig 5.6). This may reflect the increased biomass of the strain when grown in liquid culture.

Fig 3.1

DEOXYNOJIRIMYCIN AND NOJIRIMYCIN PRODUCTION BY
Streptomyces SPECIES GROWING ON SOLID MEDIA.

The strains were grown on Minimal Glucose Agar and incubated at 28°C.

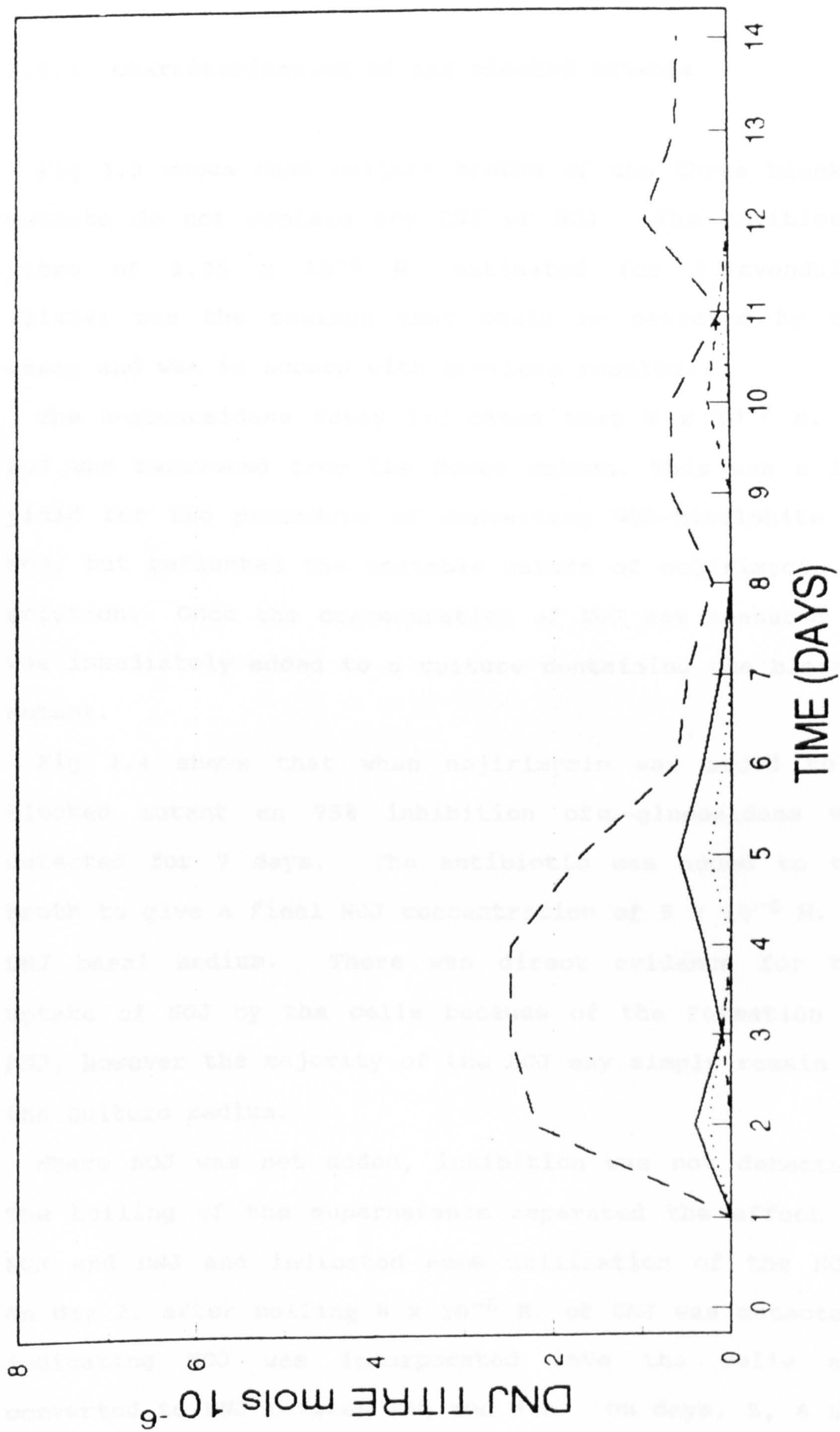


Uninoculated Agar *S. lavendulae* 8664 *S. subrutillus* *S. lavendulae* 31434

Fig 3.2

PRODUCTION OF DEOXYNOJIRIMYCIN BY Streptomyces
GROWN ON A SOLID MEDIUM.

The strains were grown on Minimal Glucose Agar and incubated at 28°C. The effect of any Nojirimycin was removed from the supernatants by boiling in 6M HCL for 6 hours.



Uninoculated Agar *S.lavendulae* 8664 *S.subnubilus* *S.lavendulae* 31434

3.2.3 Characterisation of the blocked mutants

Fig 3.3 shows that culture broths of the three blocked mutants do not contain any DNJ or NOJ. The antibiotic titre of 1.25×10^{-5} M. estimated for *S.lavendulae* (31434) was the maximum that could be detected by the assay and was in accord with previous results.

The α -glucosidase assay indicated that 9×10^{-7} M. of NOJ was recovered from the Dowex column. This was a low yield for the procedure of converting NOJ-bisulphite to NOJ, but reflected the unstable nature of nojirimycin in solution. Once the concentration of NOJ was measured it was immediately added to a culture containing the blocked mutant.

Fig 3.4 shows that when nojirimycin was added to a blocked mutant an 75% inhibition of α -glucosidase was detected for 7 days. The antibiotic was added to the broth to give a final NOJ concentration of 5×10^{-6} M. in DNJ basal medium. There was direct evidence for the uptake of NOJ by the cells because of the formation of DNJ, however the majority of the NOJ may simply remain in the culture medium.

Where NOJ was not added, inhibition was not detected. The boiling of the supernatants separated the effect of NOJ and DNJ and indicated some utilisation of the NOJ. On day 2, after boiling 8×10^{-6} M. of DNJ was detected, indicating NOJ was incorporated into the cells and converted to DNJ between 24h and 48h. On days, 5, 6 and

Fig 3.3

THE PRODUCTION OF DEOXYNOJIRIMYCIN AND
NOJIRIMYCIN BY S.lavendulae AND MUTANT
STRAINS OF S.lavendulae.

Figures were derived from continuous sampling of two replicate
flasks. There was negligible production of deoxynojirimycin
by any of the three blocked mutants.

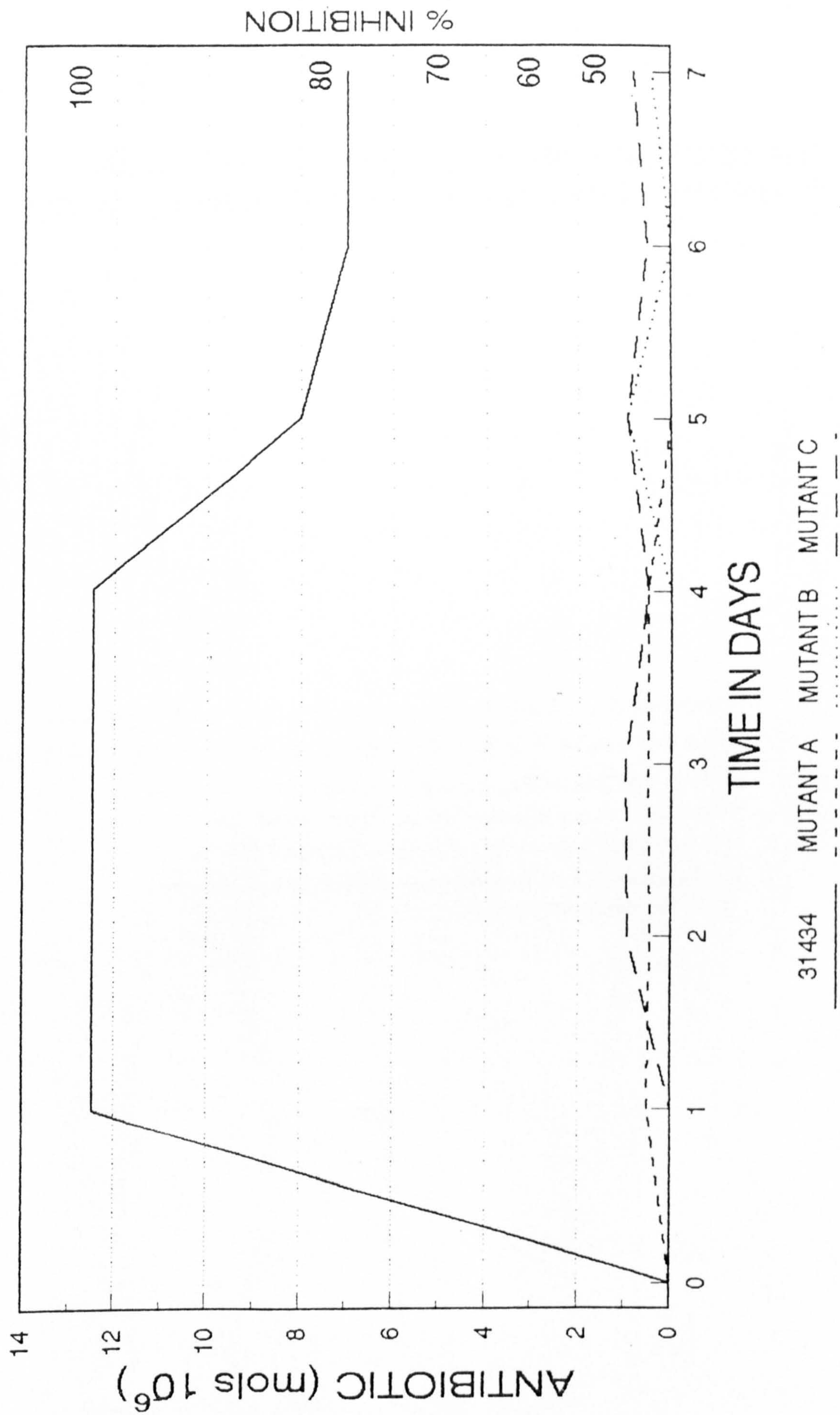
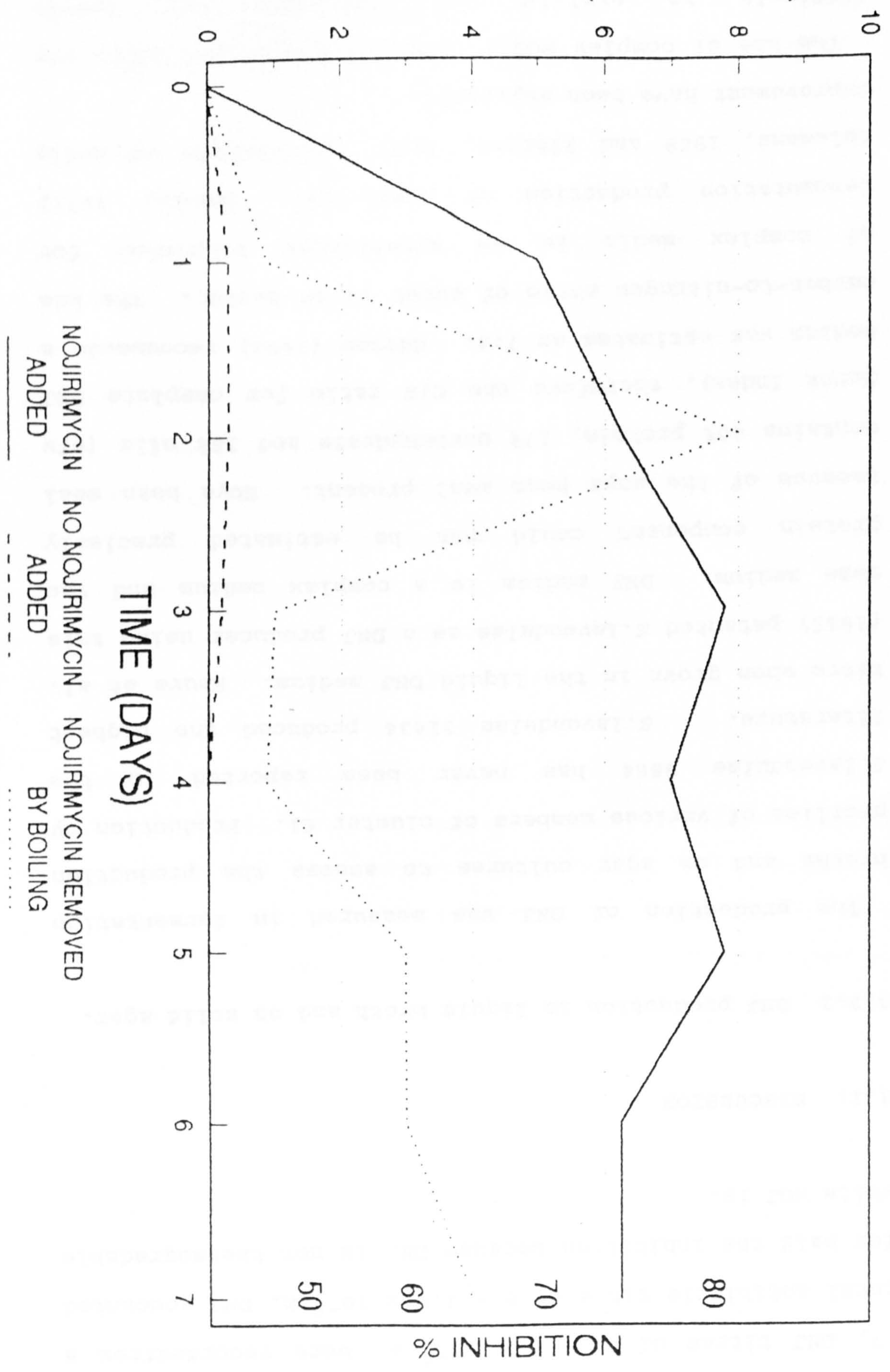


Fig 3.4

THE PRODUCTION OF DEOXYNOJIRIMYCIN AND
NOJIRIMYCIN BY S.lavendulae BLOCKED MUTANTS.

Nojirimycin added at day 1 to give a final concentration of $4.9 \times 10^{-6} \mu\text{M}$. This immediately caused inhibition compared to the sample without nojirimycin added. However, inhibition was detected in the sample where nojirimycin was removed by boiling in 6M HCl, indicating some nojirimycin had been converted to deoxynojirimycin.

DNJ AND NOJ (Mols 10^6)



7, DNJ titres of $3 - 4 \times 10^{-6}$ M. were recorded from a total antibiotic titre of $6 - 7.5 \times 10^{-6}$ M. DNJ accounted for half the inhibition because DNJ is not thermogradable while NOJ is.

3.3. DISCUSSION

3.3.1 DNJ production in liquid broth and on solid agar.

The production of DNJ was measured in fermentation broths and on agar cultures to assess the production profiles of various members of cluster 61. Production by *S.lavendulae* 8664 has never been reported in the literature. *S.lavendulae* 31434 produced the highest titre when grown in the liquid DNJ medium. Ezure et al. (1985) patented *S.lavendulae* as a DNJ producer using this same medium. DNJ medium is a complex medium and the protein component could not be estimated precisely because of the soya bean meal present. Soya bean meal contains 40% protein, 17% carbohydrate and 18% oils (w/w Merck Index), therefore the C/N ratio for complete DNJ medium was estimated as 7:1. Hütter (1982) recommends a carbon-to-nitrogen ratio of about 10 or higher. The use of complex media is an established tradition for fermentation production of antibiotic. (Booth, 1971; Solomons, 1969 and Ribbons, 1970) and methods of media improvement have been empirical.

The use of complex media rather than defined media was difficult to explain and underlines how poorly

understood, are the complex interactions between nutrients in fermentation broths. Hütter (1982) suggested that the success of complex media was due to the variety of carbon and nitrogen sources in the medium. Despite the different shift downs in nutrients which will occur during fermentation, prolonged carbon or nitrogen limitation should be avoided. The C/N ratio could be accurately determined for the other media since they were defined media. However, the whole concept of C/N ratio may not be the most significant factor determining antibiotic production. The medium with the lowest C/N ratio was minimal glucose medium (6:1) which gave the lowest titres. In this medium glucose probably suppressed the production of DNJ. When the DNJ medium contained starch as the carbon source, glucose or dextrans may have been released more slowly which could have been responsible for the elevated production. Glycerol is a three carbon substrate, however no production was detected from any of the strains when the glycerol-proline medium (C/N ratio 8:1) was used. The second most productive medium using *S.lavendulae* 31434 was ISP4 (C/N ratio 10:1) which, like the DNJ medium contained starch as its carbon source. The rate of release of carbon was possibly more important than the C/N ratio. The C/N ratio alone gives a poor correlation with antibiotic production. However, strains growing in media containing starch produced the higher antibiotic titres. Both malic acid and MGA had two carbon sources in their media, but neither yielded high titres of DNJ.

The low C/N ratio and high proportion of carbon in the malic acid media maybe responsible for repressing production.

DNJ production from *S.subbrutilus*, appeared to have a similar control mechanism as *S.lavendulae*. Productivity was approximately 80% lower than *S.lavendulae* in the DNJ and ISP4 media, while no production was detected with any other carbon source.

3.3.2 Characterisation of the blocked mutants

Fig 3.4 shows that addition of NOJ to the culture broth after 24 hours resulted in a final concentration of 5×10^{-6} M. The antibiotic titres increased on days 3 and 4 to 7 and 7.5×10^{-6} M which indicated production of additional DNJ, since the mutants by definition were blocked in the production of NOJ. The reason that there appears to be twice as much DNJ synthesised than NOJ added, on day 2, was a reflection of variation in the data. The number of replications was limited by the availability of NOJ, which can not be purchased and only a small amount was obtained from the Department of Chemistry (Warwick University). Fig 3.4 shows the NOJ and DNJ titres from one culture when NOJ was not added. There was no production of inhibitor and the inhibition values were similar to those of the mutants in Fig 3.3. This demonstrated that there was no reversion of the mutant to the wild type before or during the course of the experiment.

When the supernatants were boiled in acid the inhibition due to NOJ was removed and on day 1 the titre declined to 1×10^{-6} M. showing it was the added NOJ that caused the inhibition in the unboiled sample. On day two a titre of 8×10^{-6} M. of DNJ was estimated and indicated the mutant strain had readily converted NOJ to DNJ, in a period of only 24 hours. However, the decline of the DNJ titre to 1×10^{-6} M. suggests NOJ may be responsible for much of the inhibition on day 2. This may occur if the NOJ in the sample was not thoroughly degraded. The increase in DNJ titre on days five and seven indicated a slow accumulation due to slow conversion of NOJ to DNJ.

3.3.3 Conclusion

The α -glucosidase assay could determine titres of NOJ and DNJ from culture supernatants. This work illustrated interesting variations in species and strains within the *S.lavendulae* taxonomic cluster.

None of the blocked mutants complemented each other (Trew 1992), so more mutants would have to have been produced to do cosynthesis experiments and assess the number of enzymatic steps in the biosynthetic pathway.

However, the biochemical feeding data from the successful complementation of the blocked mutants with nojirimycin indicated a block in one of the enzymatic steps, prior to NOJ formation blocking DNJ production. No block was detected in the final step(s) converting NOJ to DNJ. The work also demonstrated that none of the mutants reverted to the wild type.

CHAPTER FOUR

THE PREPARATION OF SEVERAL GENE BANKS FOR *S.LAVENDULAE* AND *S.SUBRUTILUS*.

4.1 Introduction

S.lavendulae and *S.subutilus* both contain the biosynthesis genes for DNJ production. The genes are likely to be clustered, but the size of the gene cluster can only be estimated by examination of the number of steps in the biochemical pathway. The location of the gene cluster on the chromosome is unknown. The assumption that the genes are clustered was based on the evidence provided by successful cloning of a range of gene clusters involved in production of antibiotic biosynthesis genes; for example, actinorhodin (Malpartida and Hopwood 1984), bialaphos (Murakami et al., 1986), methylomycin (Chater and Bruton, 1983) and streptomycin (Ohnuki et al., 1985). The clustering of these genes facilitated the cloning of the entire pathway and subsequent study of production.

The effects of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) also indicated clustering of biosynthesis genes. NTG mutagenesis can be focused on a particular area and its effects directed away from undesirable chromosomal damage, in a technique called comutation. The procedure was one of the first to demonstrate that actinorhodin genes were clustered.

Successful cloning of the NOJ or DNJ production genes would provide an opportunity for improved understanding of the biosynthesis of DNJ, regulation of production and the enzymes involved in biosynthesis.

An increased understanding of the genetic basis for biosynthesis of secondary metabolites has led to new approaches for the improvement of antibiotic yields. Previous empirical mutation and selection techniques have been well used, but gene dosage is a new approach. By cloning extra copies of regulatory genes into the producing organism the structural genes can in some cases produce more antibiotic. There is also speculation as to whether late onset of antibiotic biosynthesis reflects late transcription. This leads to the possibility of bringing forward transcription by substituting vegetative for late promoters. A similar dramatic increase in antibiotic yield is predicted when carbon and nitrogen repression is better understood.

It is becoming increasingly difficult to discover novel isolates and new antibiotics. Gene cloning is one approach to achieving greater novelty. By making gene libraries of donors and cloning these into recipients, new combinations of biosynthetic genes are created, by combining two pathways. This technique relies on the substrate specificity of the biosynthetic enzymes involved. Hopwood et al. (1985) used this method when actinorhodin genes from *S.coelicolor* were cloned into pIJ922 and pIJ940. Sub-clones carrying different transcription units were introduced into *S.violaceoruber*,

a mutant of *S.violaceoruber* and *Streptomyces* spp AM-7161 (medermycin producer). Transformation efficiency was low, with only 1 to 10 transformants per μ g of plasmid DNA recorded. Despite this, all the transformations produced viable transformants and *S.coelicolor* genes were expressed in each of the recipient strains, which led to the production of novel antibiotics. Before any of these procedures could be undertaken the genes of interest had to be cloned.

4.2 RESULTS

4.2.1 Shotgun cloning and approaches to making a gene bank.

The results from the shotgun cloning are outlined in the following four sub-sections.

4.2.2 Restriction digests of the *S.lavendulae* (31434) and *S.subbrutilus* (445) chromosomes.

*Sau*3A was selected for digestion of *S.subbrutilus* DNA and after only 30 seconds some fragments were reduced to a size of only 500 base pairs. This contrasted markedly with the uncut controls. The difference between 30 and 45 second digest times was not very distinct, and fragments could be removed from all eight lanes for future use (data not shown). This method was successfully repeated with chromosomal DNA of *S.lavendulae*. This DNA had a more observable difference between the 30 and 45 second digests. The DNA digested for 45 seconds resulted in a maximum fragment size of

6kb. Since it is impossible for such fragments to contain the entire gene cluster (estimated size 10kb using kb ladder), only the fragments greater than 10kb, from the 30 second digest, were eluted from the gel.

4.2.3 DNA fragment size after gel fractionation and elution

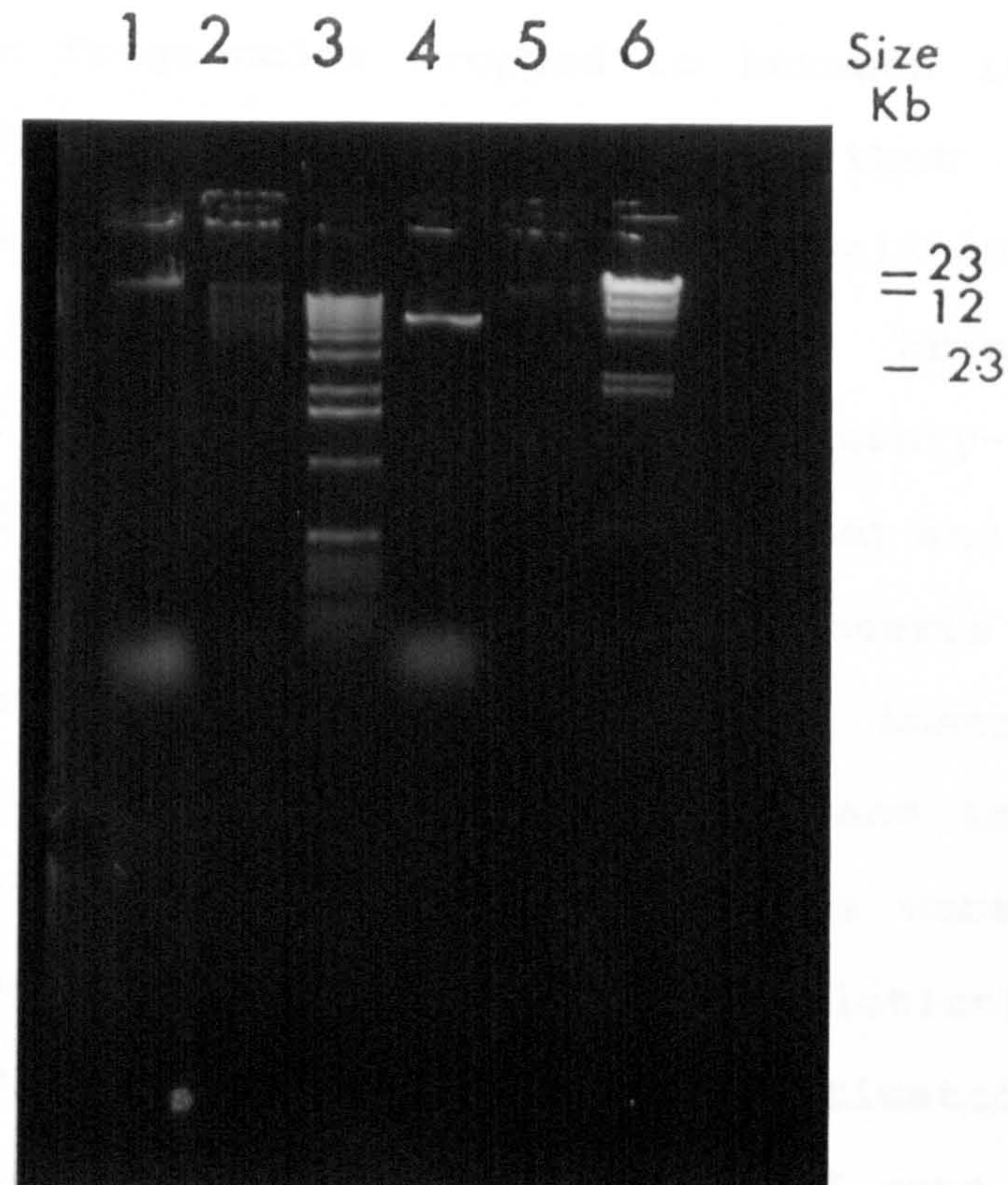
The DNA was eluted from gels containing chromosomal DNA fragments of *S.lavendulae* and *S.subbrutilus*. The fragments were almost all larger than 12kb, which should be large enough to contain the DNJ gene cluster. Fragments such as these were used in the ligations.

4.2.4 Ligation of chromosomal *S.lavendulae* into pIJ702.

Once ligation was completed, a sample of ligation mix was run on a gel to indicate the success of any given ligation. The ligation of *S.lavendulae* DNA fragments into pIJ702 is illustrated in Fig 4.1 (lane 5). This product was larger than unligated linear pIJ702, indicating that ligation had occurred. There are also no *S.lavendulae* DNA fragments visible in lane 5 indicating probable ligation into the plasmid. This plasmid band would not have been visible, at the position shown on the gel without ligation having occurred..

Fig 4.1

Ligation of chromosomal S.lavendulae into plJ702.



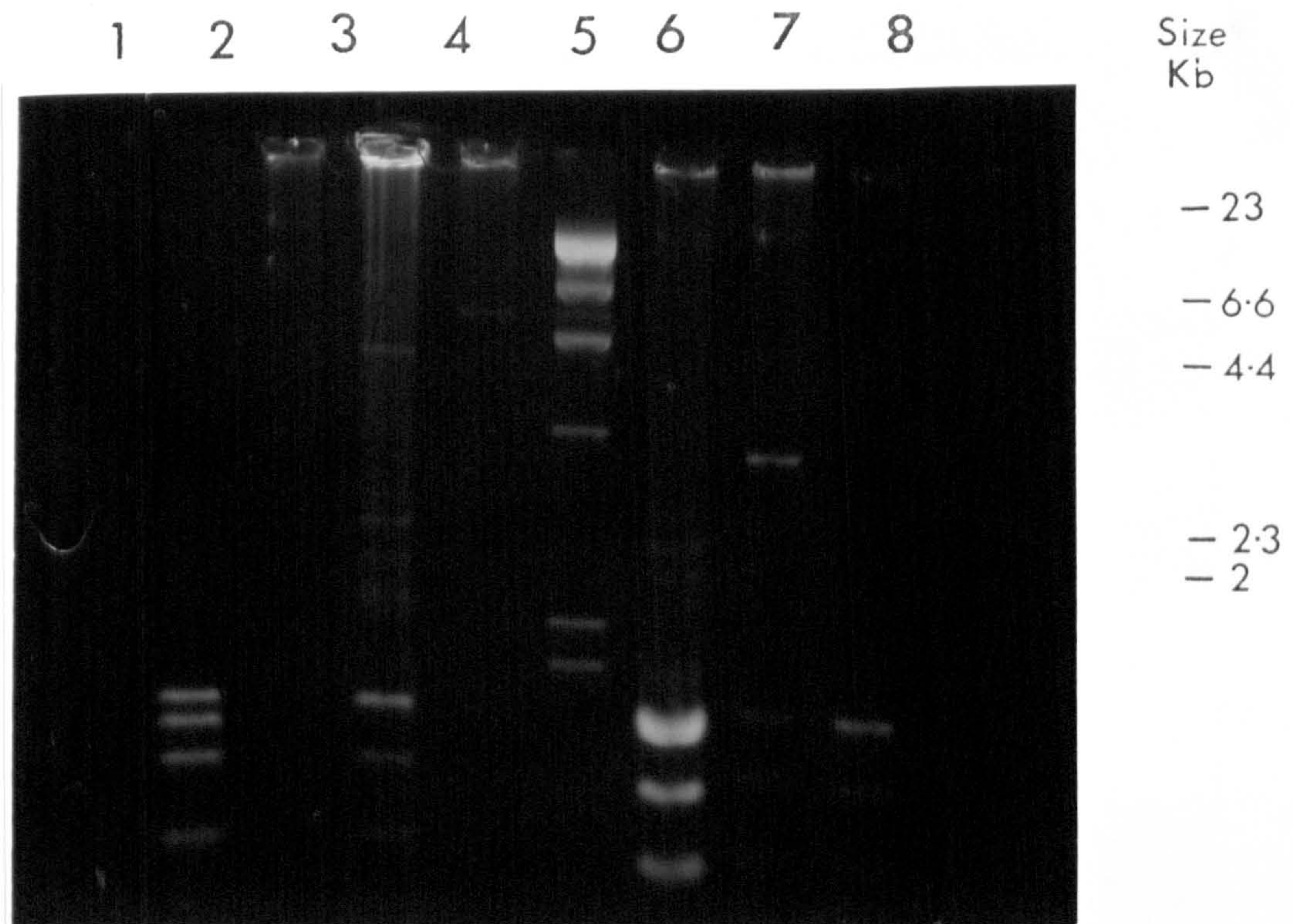
**Lane (1) Uncut chromosomal *S.lavendulae* DNA
(2) Fragments of *Sau* 3A cut *S.lavendulae*
unligated. (3) 1kb ladder. (4) Unligated
plJ702 linearised with *Bgl* II. (5) Ligation
of *S.lavendulae* DNA fragments into plJ702. (6)
Lambda Hind III digest.**

4.2.5 Transformation frequencies

Transformation of *S.lividans* with uncleaved vector gave transformation frequencies of $10^8/\mu\text{g}$ of plasmid. This produced a confluent growth of colonies through the thiostrepton overlay. When using a recombinant vector, transformation frequencies dropped to between $10^4/\mu\text{g}$ and $10^5/\mu\text{g}$ of plasmid DNA. Transformants that contained recombinant DNA were unable to produce melanin, due to insertional inactivation. Recombinant transformants (2500 in total) were isolated and twenty-one were randomly selected. Plasmid DNA was prepared and digested with *Bcl* I to determine the size of the inserts Fig 4.2. Fig 4.3 shows the frequency of different insert sizes. Ten of the inserts were smaller than 1kb and in most of those only between 100 and 200 base pairs were cloned. Only one transformant (<5% of total population) had an insert in excess of 10kb, which was the estimated minimum size of fragment that could contain the DNJ gene cluster. Hence, only an estimated 100 to 150 of the 2500 transformants could contain large enough DNA fragments. Even if the average length of the insert was 5kb, 4604 clones would be required to have a 90% probability of a complete library (Hopwood et al., 1985). Calculations based on the insert size and the known size of the *Streptomyces* genome indicated, an estimated 200,000 transformants would have to be screened to account for the whole genome, which was impractical.

Fig 4.2

**Bcl I Digest of Recombinant pIJ702 Plasmids
to Determine the size of Cloned DNA.**



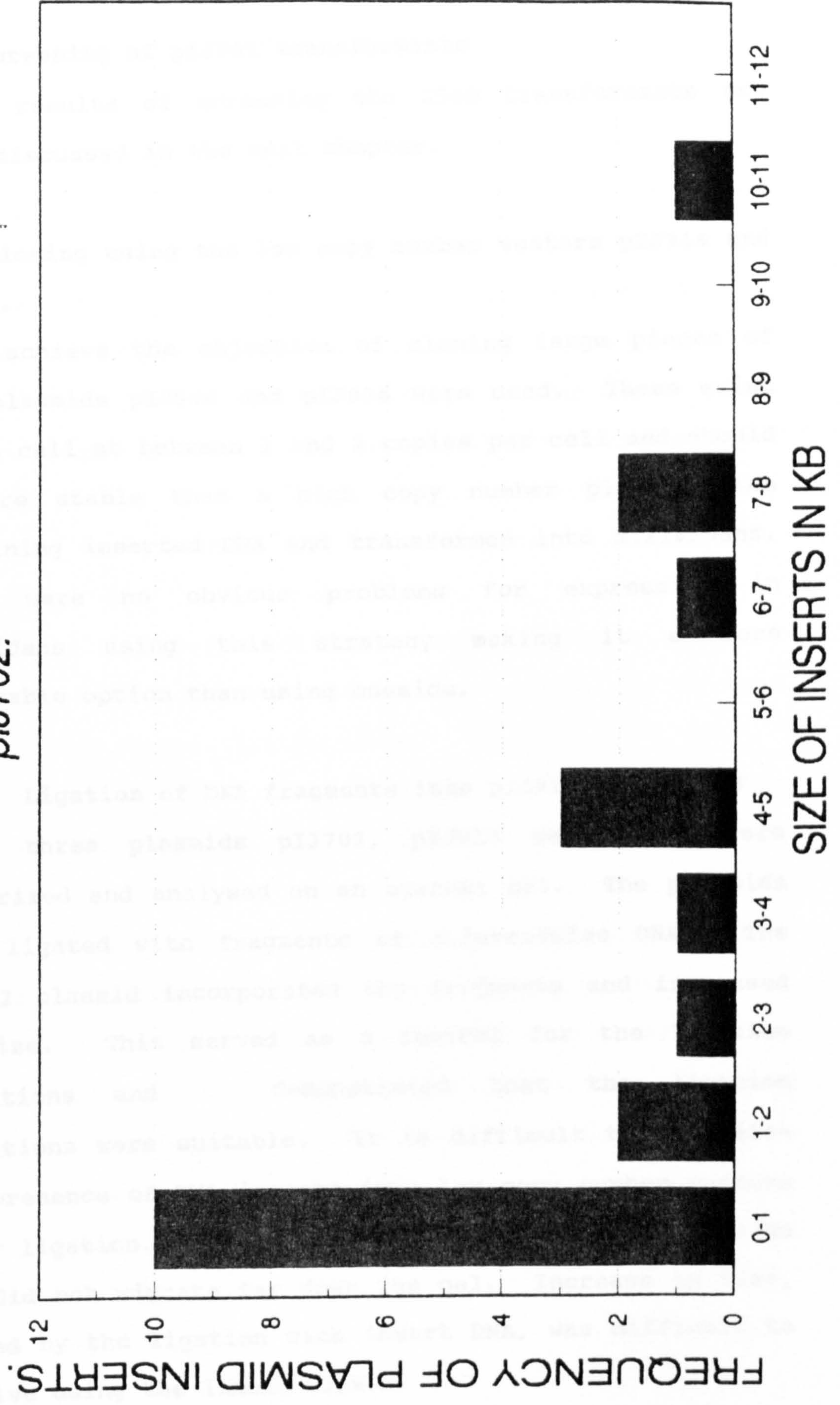
Lane (1) Bcl I digest of pIJ702. Lanes (2,3,4,6,7, and 8) Bcl I digest of prepared plasmids. Lane (5) Lambda Hind III digest.

Fig 4.3

**THE SIZE OF THE DNA INSERTS WITHIN
RECOMBINANT pIJ702.**

THE SIZE OF THE INSERTS WITHIN RECOMBINANT

plJ702.



4.3 Screening of pIJ702 transformants

The results of screening the 2500 transformants are fully discussed in the next chapter.

4.4 Cloning using the low copy number vectors pIJ916 and pIJ940.

To achieve the objective of cloning large pieces of DNA, plasmids pIJ940 and pIJ916 were used. These exist in the cell at between 1 and 2 copies per cell and should be more stable than a high copy number plasmid when containing inserted DNA and transformed into *S.lividans*. There were no obvious problems for expression in *S.lividans* using this strategy making it a more favourable option than using cosmids.

4.4.1 Ligation of DNA fragments into pIJ916 and pIJ940

The three plasmids pIJ702, pIJ916 and pIJ940 were linearized and analysed on an agarose gel. The plasmids were ligated with fragments of *S.lavendulae* DNA. The pIJ702 plasmid incorporated the fragments and increased in size. This served as a control for the ligation conditions and demonstrated that the ligation conditions were suitable. It is difficult to determine the presence of DNA inserts into low copy number vectors after ligation. The non-recombinant plasmids were large and did not migrate far down the gel. Increase in size, caused by the ligation with insert DNA, was difficult to resolve using the lambda marker.

4.4.2 Transformations using pIJ916 and pIJ940

Four combinations of transformations were carried out, using the two different vectors, pIJ916 and pIJ940 and the two types of host DNA, *S.lavendulae* and *S.subbrutilus*. A difference in the number of transformants produced from cleaved and uncleaved vector was clearly visible in Fig 4.4. The number of transformants produced after transformation with ligation mix which showed evidence of ligation having occurred, was approximately ten times less than transformants produced from ligation mix containing only uncleaved vector. The number of transformants produced for each library is shown in Table 4.1. The 2500 transformants produced using pIJ702 were included for comparative purposes.

There was a higher frequency of transformants using *S.lavendulae* DNA, with a total of 1700 transformants compared to 200 for *S.subbrutilus*. The number of transformants produced from pIJ916 and pIJ940 were similar. Unlike the pIJ702 there is no equivalent of melanin inactivation with either pIJ916 or pIJ940. It was therefore impossible to differentiate between *S.lividans* transformed with a non-recombinant or recombinant plasmid. The only way to determine the size of an insert was to extract the plasmid and subject it to a restriction digest.

Fig 4.4

The difference in the number of transformants produced by transformation with recombinant and uncleaved vector.

Fig 4.4

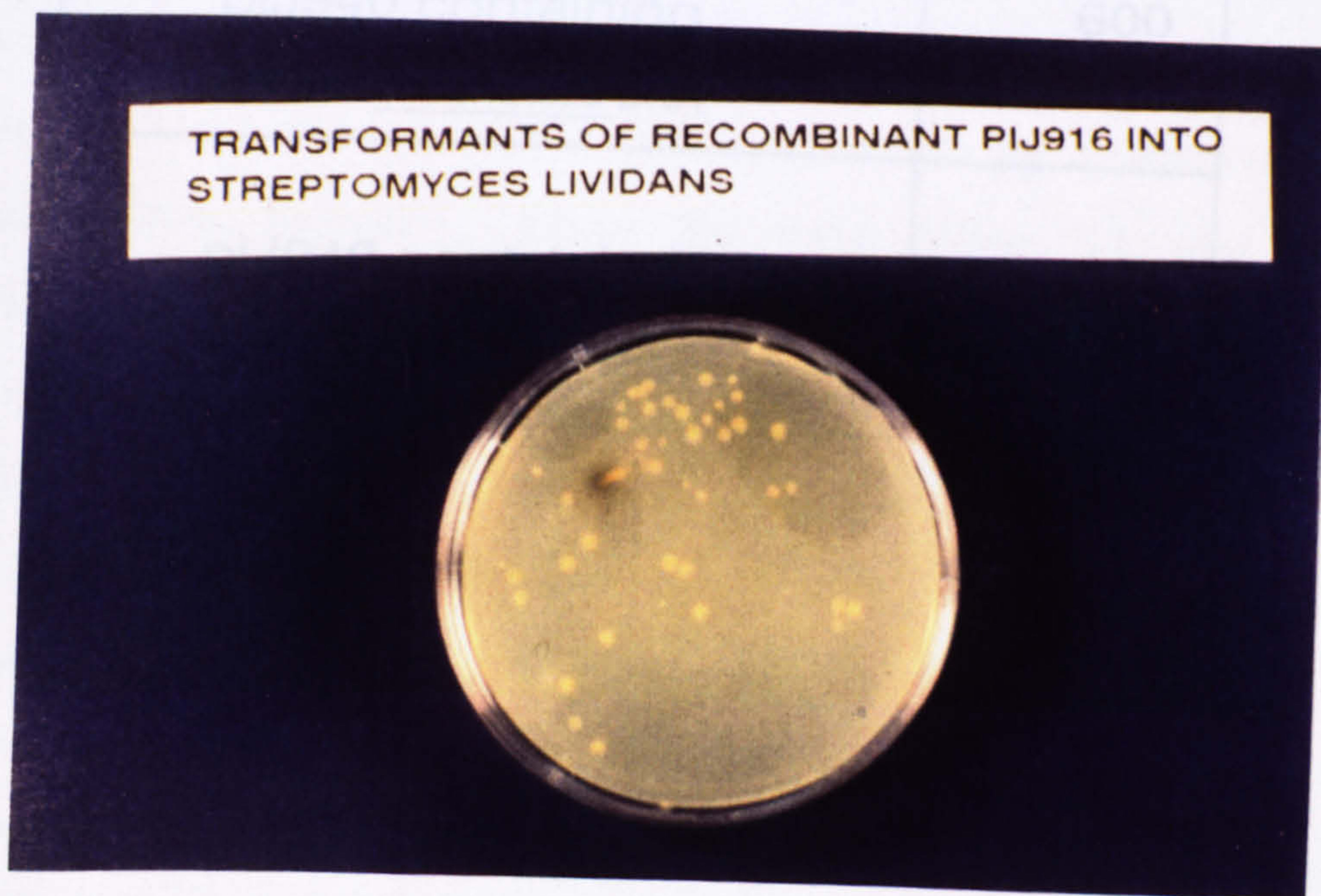
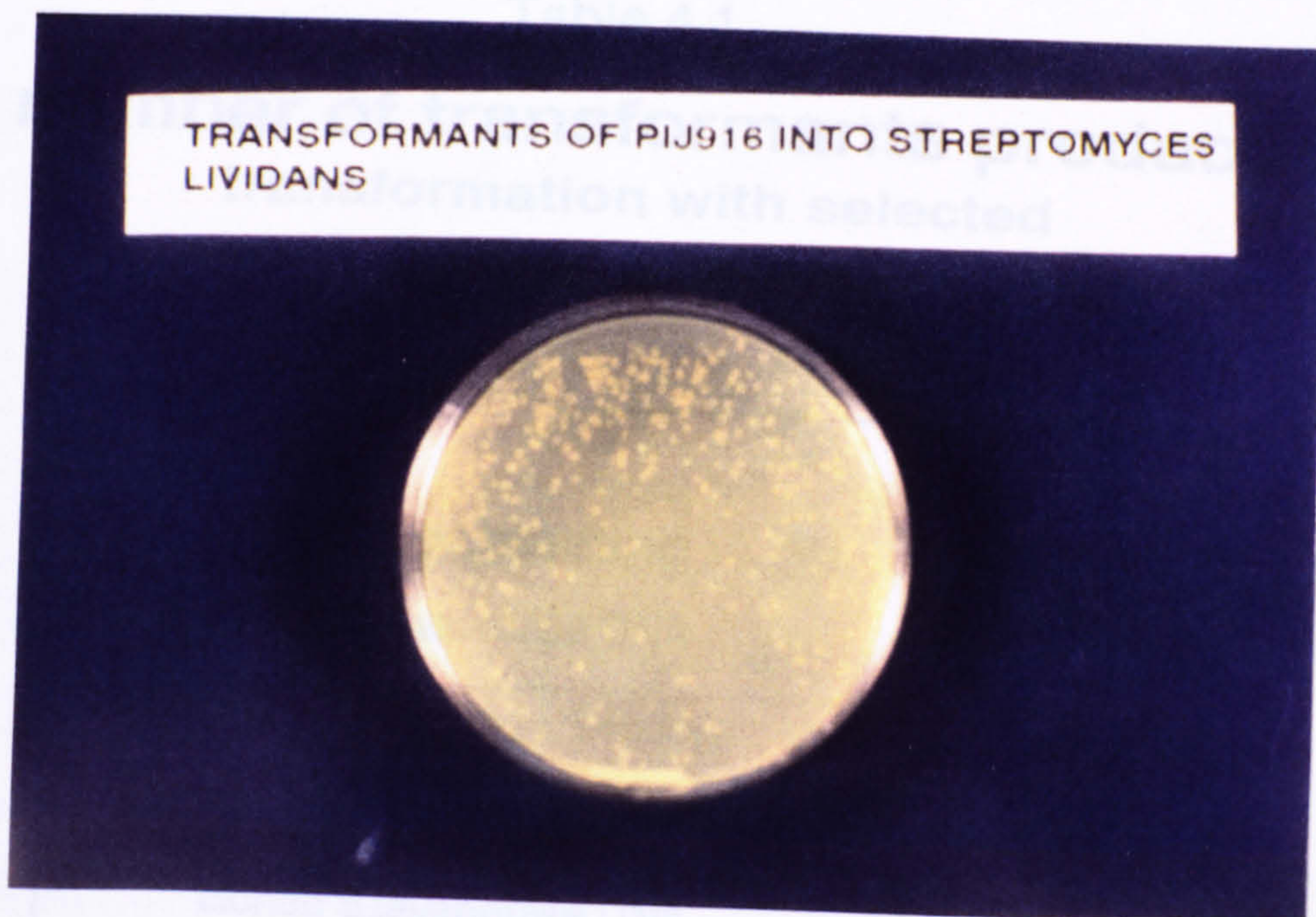


Table 4.1

The number of transformants produced by transformation with selected ligation mixtures.

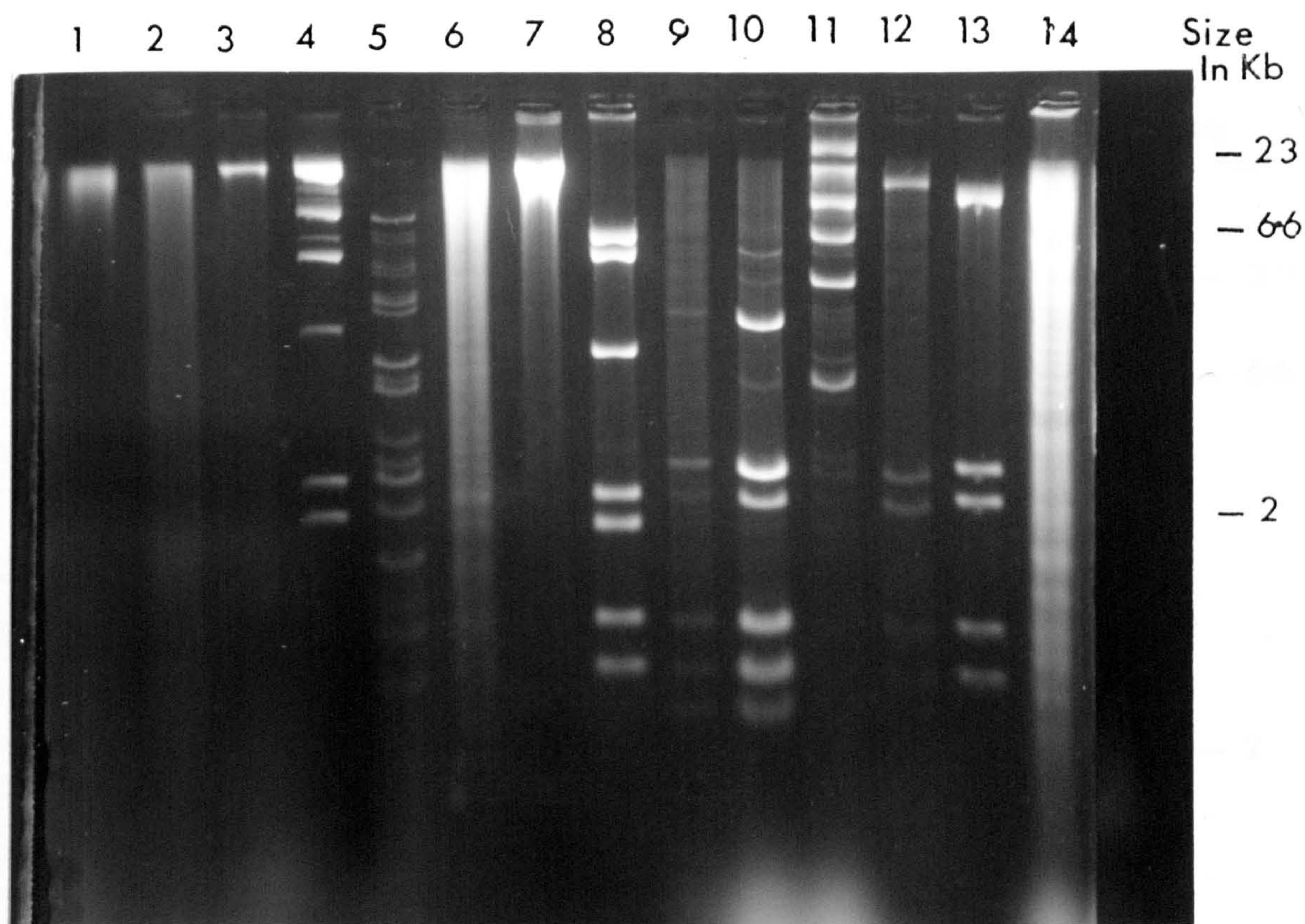
plJ702 containing cloned <u>S.lavendulae</u> DNA	2,500
plJ916 containing cloned <u>S.lavendulae</u> DNA	1,100
plJ916 containing cloned <u>S.subtrillius</u> DNA	60
plJ940 containing cloned <u>S.lavendulae</u> DNA	600
plJ940 containing cloned <u>S.subtrillius</u> DNA	150

4.4.3 Restriction digests of pIJ916 and pIJ940 recombinant plasmid.

Restriction digests of six recombinant pIJ916 and pIJ940 plasmids (from a population of 1800) are shown in Figs 4.5 and 4.6. In Fig 4.5 lanes 9, 10 and 11 were digests of recombinant pIJ940 and were run adjacent to the pIJ940 wild type plasmid which acted as a control. Lanes 1-5, 7 and 8 were concerned with pIJ702 and were discussed in section 4.2.5. In Fig 4.5 digests of recombinant pIJ940 were run in lanes 5 and 6. These digests are not as clear as in Fig 4.6. However, lanes 9, 10 and 12 contain digests of pIJ916 run adjacent to the pIJ916 control plasmid. The sizes of 8 digested recombinant plasmids were 12kb, 6kb, 6kb and 25kb for pIJ916 and 7.2kb, 8.7kb, 6.1kb and 26kb for pIJ940. The data for the two additional digests came from minipreps. The size of the plasmids was estimated by comparison with the lambda marker. From the data shown it was obvious that substantial deletions had occurred. In six of the eight plasmids DNA of size 7kb to 19kb was deleted and two of the plasmids were not deleted. The *Bgl* II site, which was the insertion site for DNA on pIJ940 and pIJ916, was located on a 7.32kb and a 12.07kb *Bcl*I fragment respectively. For the undeleted plasmids there was evidence of insertions into these sites. The pIJ916 *Bcl*I band containing the insertion site for cloned DNA increased in size from 12.07kb to at least 19kb.

Fig 4.5

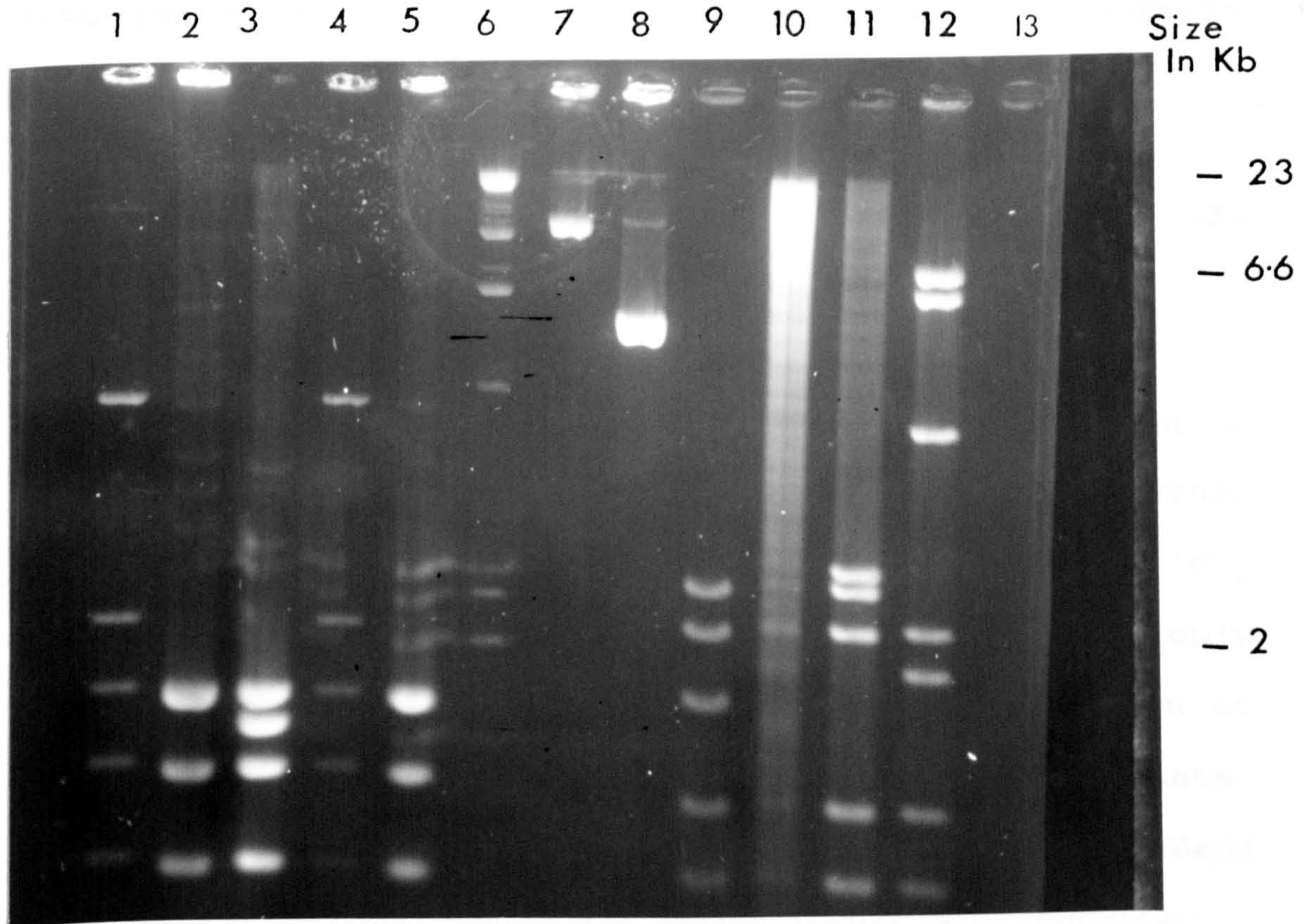
BclI Digest of Recombinant pIJ702, pIJ916 and pIJ940 to determine the quantity of cloned DNA.



*Lanes (1-3) DNA Prepared from Caesium Gradients.
Lane (4) Lambda Hind III digest. Lanes (5-7) Bcl I digest
of recombinant pIJ940. Lane (8) digest of pIJ940.
Lanes (9-12) digests of recombinant pIJ916. Lane
(13) digest of pIJ916. Lane (14) digest of S.lavendulae.*

Fig 4.6

Restriction Enzyme Digest of Recombinant plJ702 and plJ940 to determine the size of cloned DNA.



Lanes (1-5) are Bcl I digests of recombinant plJ702.

Lane (6) Lambda Hind III digest. Lanes (7-8)

Eco RV digest of recombinant plJ702. Lanes

(9-11) Bcl digests of recombinant plJ940.

Lane (12) Bcl I digest of plJ940.

The precise amount of DNA incorporated into the plasmid is difficult to determine, because the small distance between bands could not be measured accurately. The pIJ940 *Bcl* I band containing the insertion site for cloned DNA increased in size from 7.32kb to approximately 12kb (data not shown) and demonstrates that DNA inserts were cloned using both of these vectors.

4.5 Transformation frequencies using the ZX1 modification mutant.

The ZX1 protoplasts produced confluent regeneration of transformants when transformed with uncleaved pIJ702. The frequency per μg plasmid DNA was approximately 10^8 . Frequencies using recombinant pIJ702 were much less, only 380/ μg plasmid DNA were recorded. Closer examination of these colonies revealed they were spontaneous mutants. Plasmid preparations were performed using the standard plasmid "miniprep" method and no plasmids were detected.

Transformation frequencies were between 10^4 and 10^5 per μg plasmid DNA when transformed with uncleaved pIJ916. Transformation frequencies using the wild type *S.lividans* were approximately 3 times greater at 10^5 per μg plasmid DNA. However, frequencies of recombinant pIJ916 were $10^5/\mu\text{g}$ plasmid DNA, which was the same as would be expected using the wild type *S.lividans* protoplasts. This indicated that use of the zx1 mutant had no deleterious effect on transformation frequencies.

4.6 Probing plasmid restriction digests with labelled pIJ940

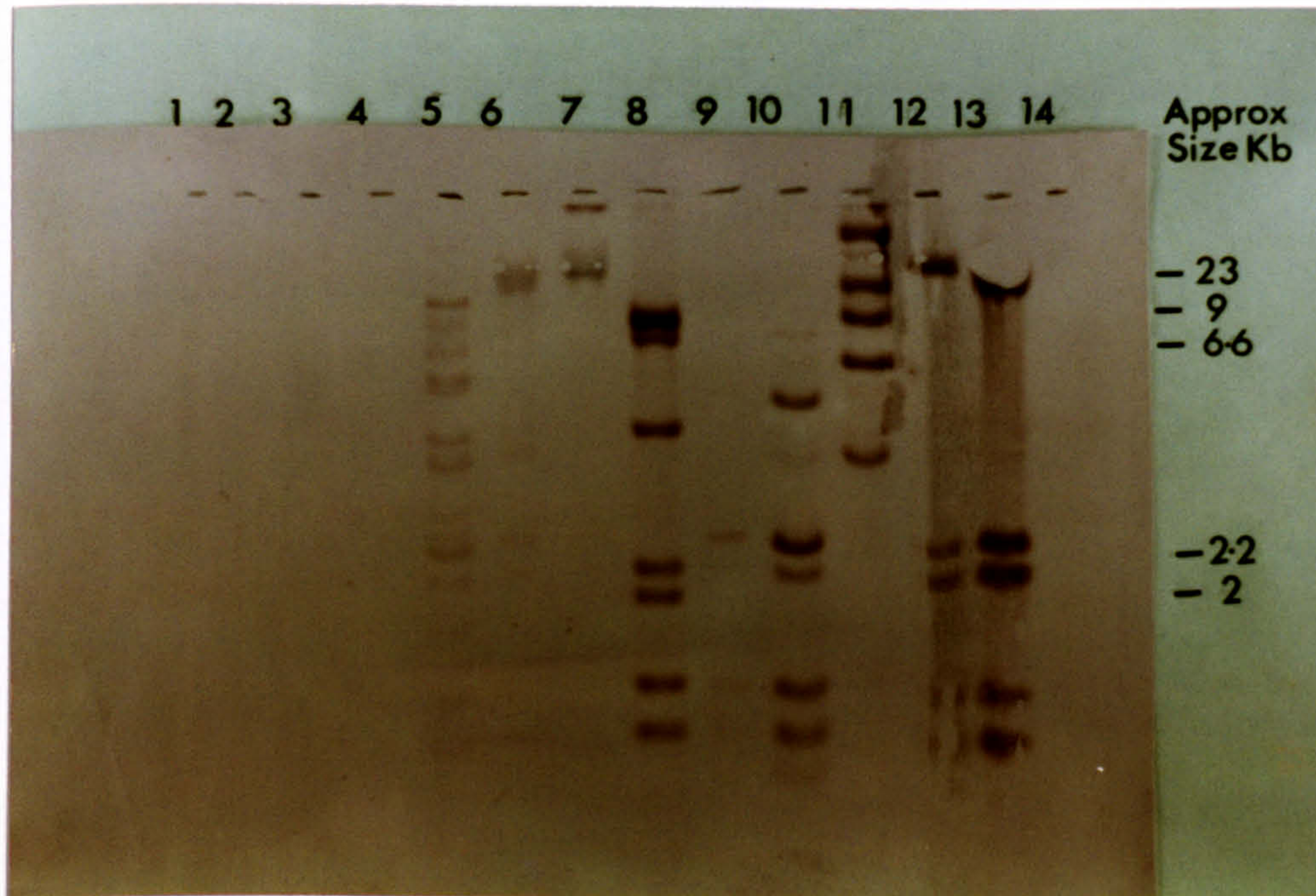
The aims of the hybridisation experiment in Fig 4.7 were threefold. Firstly, to determine if the DNA in lanes 1-3 was of plasmid or chromosomal origin. Secondly, to assess the size of the plasmid that was restricted in lane 6 and finally determine the origin of the DNA bands from the digested plasmids in lanes 5-11.

The positive controls, in lanes 8 and 13 (restricted pIJ916 and pIJ940) both provided clear positive bands. The *S.lavendulae* negative control in lane 14 produced no bands, indicating it had no homology with the DNA probe (labelled pIJ940) at a stringency of 88%.

The probing was very effective at confirming whether the isolated DNA (lanes 1-3) was plasmid or chromosomal. No bands were visible, so only chromosomal DNA had been isolated and the samples were discarded. The size of the digested plasmid in lane 6 was estimated to be 8.7kb. The diffuse band running level with the lambda marker was uncut DNA, below which were 5 visible bands. Prior to probing it was impossible to determine the size of this restricted plasmid because chromosomal DNA from *S.lividans* obscured the plasmid bands. When Fig 4.7 was compared to Fig 4.5 all the digests produced positive bands with the probe.

Fig 4.7

PROBING PLASMID RESTRICTION DIGESTS
WITH pIJ940 LABELED WITH THE DIGOXIGENIN
NONRADIOACTIVE DNA LABELING AND
DETECTION KIT.



Lanes 1-3 DNA from transformants.

Lane 4, Hind III Lambda digest. Lanes

5,6,9,10 and 12 recombinant plasmids cut with
Bcl I. (pharmacia) Lanes 7 and 11 recombinant
plasmids cut with Bcl I (BRL). Lane 8 and 13
pIJ940 and pIJ916 wild type plasmids.

Lane 14, chromosomal DNA from S.lavendulae.

4.7 Restriction systems within *Streptomyces*

To examine the restriction system in *S.lividans* and *S.lavendulae* KC301 (a derivative of ϕ C31 with a thiostrepton marker gene) lysates were made by transfecting *S.lavendulae* and *S.lividans*. When *S.lividans* lysate was titred upon a lawn of *S.lividans* cells, confluent lysis was seen even when the lysate was diluted 10^{-5} (Table 4.2). It was only possible to count plaques and hence estimate phage numbers (8.9×10^7) on one replicate at the 10^{-5} dilution. The same lysate titred on lawns of *S.lavendulae* only produced confluent lysis when the lysate was diluted 10^{-2} . It was possible to count plaques and estimate phage numbers when the lysate was diluted 10^{-3} (1×10^6 and 8.2×10^5) and even at 10^{-5} (4×10^6 and 1.6×10^7) indicating an approximate two logarithm drop in phage numbers due to host-controlled restriction in *S.lavendulae*. However, lysate from *S.lavendulae* on lawns of *S.lividans* caused confluent lysis at a dilution of 10^{-3} . Similar numbers of phages at dilutions of 10^{-4} and 10^{-5} were estimated from lawns of *S.lividans* and *S.lavendulae*, which implies there was no host-controlled restriction system in *S.lividans*.

Table 4.2

ANALYSIS OF A RESTRICTION SYSTEM BETWEEN S.lividans AND S.lavendulae.

DILUTION FACTOR	<u>S.lavendulae</u> Phage Lysate	<u>S.lividans</u> Phage Lysate	<u>S.lividans</u> Phage Lysate	<u>S.lavendulae</u> Phage Lysate
	LAWN OF <u>S.LAVENDULAE</u>	LAWN OF <u>S.LAVENDULAE</u>	LAWN OF <u>S.LIVIDANS</u>	LAWN OF <u>S.LIVIDANS</u>
10^0	C	C	C	C
10^1	C	C	C	C
10^2	C	C	C	C
10^3	C	9.1×10^5	C	7.2×10^5
10^4	5×10^6	3.7×10^5	C	1.4×10^6
10^5	7×10^6	1.0×10^7	8.9×10^7	3.0×10^6

C- Denotes confluent lysis and counts are the mean of two replicates.

4.8 DISCUSSION

4.8.1 High copy number plasmids

It was possible to speculate on several alternative explanations for the failure of the strategy using pIJ702. Certain sections of cloned DNA could have been lethal to *S.lividans* and as the cloned section of DNA becomes larger, so does the chance of incorporating a lethal gene, which leads to cell death. When the plasmid established itself at high copy number, the cloned DNA could have been transcribed and translated. Protein products, or intermediates of biochemical pathways, influenced by these products, could have been toxic to the cell. These could have caused immediate cell death, or there could have been an excision mechanism to remove sections of recombinant DNA from the plasmid. This would result in a series of spontaneous deletions from the initial recombinant plasmid, rendering the plasmid non-toxic to the cell. Only plasmids with small sections of cloned DNA would tend to survive transformation. Additionally, there would have been some stability advantage for plasmids with smaller inserts.

Homologous recombination could also have occurred between the cloned insert and the chromosome. Incomplete recombination may have destabilised some plasmids and resulted in spontaneous deletions.

The transformants obtained are the product of extreme

biological selection pressure. Initially, there may have been a large population of plasmids containing different size inserts of DNA from the ligation. Most of these plasmids could have contained a lethal quantity of DNA when they entered the protoplast at concentrations of $10^9/\text{ml}$. The 10^5 transformants $/\mu\text{g}$ of plasmid DNA that survived were the result of selection pressure for plasmids with small inserts or contained plasmids with deleted but stable inserts.

4.8.2 Low copy number plasmids

Analysis of the deleted plasmids indicated some interesting features. Lanes 5 and 7 of Fig 4.5 are pIJ940 digests. Lane 6 is also a pIJ940 digest but evidence of the size of the plasmid comes not from the gel photograph but from hybridisation analysis discussed later Fig 4.7. One important feature was the stability of the deleted plasmids. This was demonstrated by being able to prepare the plasmids from their transformed hosts. They were estimated to be 6.1kb, 7.2kb and 8.7kb in size and closer examination revealed sections of plasmid DNA in common with each digest. They each have the band sizes 1kb, 1.2kb and 2kb. The last two bands correspond to the replication functions of the plasmid and the 1kb section corresponds to the thiostrepton resistance gene. The deleted plasmids were stable entities carrying thiostrepton resistance and capable of independent existence and replication. However, as

cloning vectors they are ineffective and merely contribute to the number of transformants carrying very little or no cloned DNA. Examination of lanes 9, 10 and 12 of Fig 4.5 show that these three bands are common to digests of pIJ916. Plasmid deletion was almost certain to have been random. However, the plasmids found all contained the replication function needed for replication and the thiostrepton gene as this was selected for. Most had lost virtually all insert DNA as this was cloned into non-essential or dispensible regions.

These problems are similar to those encountered by Woodman et al. (1991) who attempted to clone the thiostrepton resistance genes of *S. azureus*, using pKC505 and SCP2 derived vectors. Results of single and multiple digests indicated that two resistance genes were located on a 12kb *Pst* I DNA fragment approximately 6kb apart from one another on the chromosome. However, when the vectors were transformed into *S. lividans* deletions occurred and only high copy number plasmids could be isolated. The plasmids contained the pIJ101 replicon and the thiostrepton resistance gene. The data suggested that both of the thiostrepton resistance genes of *S. azureus* were located on pIJ101-like plasmids that were integrated into the chromosome. This was demonstrated when probing *S. azureus* with the pIJ101 part of the plasmid containing the replication function. Of the recovered plasmids 75% were 6.7kb, whilst 25% were 5kb. The plasmids were stable but different from the conventional high copy number plasmids pIJ702 and pIJ350.

The whole cloning strategy presented in this project was somewhat inefficient. It appears that 75% of transformants were subject to deletions. The difficulty of isolating these recombinant plasmids was further evidence of the inefficiency of this strategy. Woodman et al. (1991) also found it difficult to re-isolate plasmids when pANT423 was transformed into *S.lividans*. It was asserted that the plasmids became incorporated into the chromosome by homologous recombination and could not be extracted. The fact that the 6.75kb plasmid pANT423 was often integrated into the chromosome region adjacent to thiostrepton resistance, supports the assertion.

It is possible that spontaneous mutation accounted for a proportion of the transformants. This could have been tested for by plating pIJ940 transformants on hygromycin agar. The plasmid confers resistance to the antibiotics, hygromycin and thiostrepton, so resistance to hygromycin would have confirmed transformants as genuine. However, there were no small, poor-growing transformants Fig 4.4 characteristic of spontaneously resistant colonies. Spontaneous thiostrepton resistance could also have been checked, if required, by probing with the thiostrepton resistance gene on its own.

Spontaneous mutation could also have occurred during protoplast formation and regeneration. Hopwood et al. (1983) demonstrated that these processes accounted for plasmid deletions. In a similar study Furumai et al. (1982) observed plasmid deletions and subsequent loss of

production of aureothricin in *S.kasugaensis*. Furthermore, the deletions in *S.fradiae* accounted for the loss of expression of many biosynthetic enzyme reactions and resistance to several antibiotics (Baltz and Stonesifer, 1985). The plasmids containing deletions produced during the creation of the various gene libraries could have been caused by this mutagenic process.

There are some obvious parallels between the failure of this cloning strategy and the strategy using pIJ702. However, in the strategy using low copy number vectors the situation is more complex. Common to both methods was the enormous selection pressure imposed after transformation. Plasmids carrying large inserts of cloned DNA very rarely survived. Examination of the restriction digests of pIJ702 using *BclI* revealed that the plasmid survives unchanged. It was always the 1.56kb band that showed an increase in size. pIJ916 and pIJ940 suffer substantial deletions of the plasmid sequences. It was possible that restriction enzymes inside *S.lividans* excise the cloned DNA along with sections of the plasmid. However, it was more probable that there was selection against recombinant plasmids once they have entered the cell. The selection pressure favoured smaller deleted plasmids.

To clarify this process and determine the origin of the bands of the digested plasmids probing was undertaken using labelled pIJ940. In lanes 5,9,10 and 12 any band present on Fig 4.5 but absent from the blot, would be suggestive of cloned *S.lavendulae* DNA. However, all the

bands were visible on the blot which confirmed that all the bands contained an element of plasmid DNA. There was no single band consisting solely of *S.lavendulae* inserted DNA.

4.8.3 Transformations using the ZX1 modification mutant.

Wild type *S.lividans* DNA was susceptible to double stranded cleavage during electrophoresis, when buffers contained ferrous iron (Zhou et al., 1988). The cleavage was site specific, affecting both chromosomal and plasmid preparations, fragment size from the limited digestion was approximately 6kb. It was possible that the appearance of deleted plasmids and small DNA inserts was a result of this degradation. The ZX1 mutant lacks the modification and may result in better gel preparations. ZX1 was a mutant derived by NTG mutagenesis of the recombinant deficient (Rec-) strain JT46. The ZX1 retains the Rec- phenotype of JT46 and lacks any amplified DNA segments often seen in *S.lividans* variants.

The transformation frequencies indicate no overall reduction when using ZX1. Frequencies using uncleaved pIJ702, pIJ916 and recombinant pIJ916 were all similar to those obtained with wild type *S.lividans*.

The colonies produced from recombinant pIJ702 were thiostrepton mutants. The colony morphology was much smaller and paler than transformant colony form. The frequency was also very low. The equivalent frequency of transforming recombinant pIJ702 into wild type *S.lividans*

was between 10^4 and 10^5 per μg plasmid DNA. The lack of plasmid in any of the "miniprep" preparations confirmed these colonies as spontaneous thiostrepton resistant mutants.

Conversely, the colonies produced from transformations with pIJ916 ligation mixture which indicated that ligation had occurred, were genuinely thiostrepton resistant. They were produced at high frequency (too many to be accounted for by mutation) and the colony morphology was larger. Constraints of time prevented any analysis of the size of the DNA inserts within pIJ916.

The problems associated with *Streptomyces* genetics are complex and analysis of transformants by restriction digests showed many to contain only small inserts of recombinant DNA. A restriction-modification system was the most plausible explanation for these small inserts of DNA and the plasmid deletions.

4.8.4 Restriction systems in *Streptomyces*.

Analysis of the recombinant pIJ702 DNA found many with small DNA inserts (50% contained <1kb). The population of pIJ916 and pIJ940 recombinants contained many plasmids with deletions (75%), which indicated that a restriction system may be operating in *S.lividans*. Transfections of phage from lysates of *S.lividans* to lawns of *S.lividans* and *S.lavendulae* revealed the classic restriction pattern with a 100-fold reduction of phage titre after infection of *S.lavendulae* 31434. The phage from the *S.lividans* lysate was degraded by restriction endonucleases in *S.lavendulae*. A phenomenon first observed by Dussoix and Arber (1962) working with *E.coli* and phage lambda.

Examination of the lysate from *S.lavendulae* and transfections of lawns of *S.lividans* and *S.lavendulae* did not demonstrate a similar pattern of restriction. Building up the phage population initially through transfection of *S.lavendulae* would result in the methylation of the phage DNA against *S.lavendulae* endonucleases. Since the titres from the two lawns were similar, it appeared that *S.lividans* does not have any endonucleases to degrade the transfected DNA. Additionally KC301 is a genetic tool with *S.lividans* usually the primary host for transfection because *S.lividans* has no traditional host-restriction system.

A similar experiment by Gaudreau et al.(1992) analysed the restriction of phage JHJ-1 to assess restriction in

the genus *Saccharopolyspora*. JHJ-1 grown on *S.hirsuta* 367 IC-11 was restricted by all other strains. JHJ-1 grown on *S.hirsuta* NRRL B-5792 produced identical results except that no restriction was observed in *S.hirsuta* 367 IC-11. Phage SE-3 was not strongly restricted in *Saccharopolyspora* whereas phages FRb-P and FRa-C were restricted in most strains except *S.hirsuta*. Of all the phage tested *S.hirsuta* 367 IC-11 had the weakest restriction system. The exocyttoplasmic effect of DNases was investigated as a possible cause of restriction. However, the influence of these DNases was discounted, when no DNase activity was found if strains were grown on Tryptone Soya agar.

S.coelicolor is the best genetically characterised strain of *Streptomyces* and is the most frequent host for homologous cloned DNA. However, *S.coelicolor* also possesses an effective restriction system against DNA from *E.coli*. The restriction system in *S.coelicolor*, is probably a methylation-dependant system, where every endonuclease possessed by the cell has a methylase which protects the DNA by modifying a sequence of 4-5 bases. More than 600 restriction endonucleases and 98 methylases are known (McNeil, 1988). McNeil (1988) also used the technique to clone into *S.avermitilis* which has an effective methylation-dependant restriction system. *S.avermitilis* specifically recognises and restricts plasmid DNA containing N⁶-methyladenine or 5-methylcytosine. Many other *Streptomyces* have methyl restriction either restricting N⁶-methyladenine or 5-

methylocytosine-containing DNA. However, of the strains investigated by McNeil (1988), two strains, *S.bikiniensis* and *S.fervens* had no methyl-dependent restriction system.

S.lavendulae probably has a restriction and methylase system, while *S.lividans* from the data presented in Table 4.2 appears to have no restriction system. This explains the widespread use of *S.lividans* for heterologous cloning. Commenting on the usefulness of *S.lividans* as a host for cloning Hopwood (1981) states, "*S.lividans* is largely non-restricting and has the added advantage that it can be used as an intermediate host for cloned DNA manipulated in *E.coli* and destined for *S.coelicolor*." *S.lividans* has also been utilised as a recipient of *E.coli* DNA. (Mazodier et al., 1989) transformed *E.coli* plasmids pPM801 and pPM803 in *S.lividans* by inter/generic conjugation. Despite this evidence there are still recognised restriction problems with *S.lividans*. JT46 a Rec⁻ derivative of *S.lividans* was much more stable when multicopy vectors were transformed into it, than the wild type *S.lividans*. There was no derived benefit transforming low copy plasmids derived from SCP2 into this mutant because the mutant prevents interplasmid recombination but not homologous recombination with the chromosome.

Matsushima and Baltz (1985) stated that *S.ambofaciens* and *S.griseofuscus*, were non-restricting hosts giving good transformation frequencies comparable to *S.lividans*, but their use is not widespread.

The results did not provide a positive explanation why

so many small inserts were obtained using the pIJ702. The lack of a restriction system in *S.lividans* indicated that the restriction against plasmids with larger inserts was more complex than a simple restriction-modification system. Despite these problems many transformants were produced and these underwent an intensive screening program, details of which are presented in Chapter 5.

CHAPTER FIVE

DEVELOPMENT OF A SENSITIVE AND RAPID DETECTION SYSTEM FOR DEOXYNOJIRIMYCIN AND NOJIRIMYCIN BASED ON INHIBITION OF α -GLUCOSIDASE

5.1 Introduction

A number of different assay methods were available to detect DNJ; bioassays against whole organisms, bioassays using agar plate screens involving enzyme inhibition, colorimetric systems and assays using microtitre plate technology. Use of a whole organism bioassay would only have been applicable if the targeted antibiotic had been NOJ. NOJ is active against *Shigella flexneri*, *Sarcina lutea* (*Micrococcus luteus*) and *Xanthomonas oryzae* (Ishida et al., 1967) however, DNJ does not inhibit microbial growth and the primary objective of the assay system was to detect clones producing DNJ.

Attention was then focused on other enzyme assay systems for detecting DNJ. To screen many transformants a plate screen was devised based on the inhibition of amyloglucosidase (E.C. 3.2.1.3) by DNJ. Amyloglucosidase was incorporated with starch into an overlay. DNJ production by a colony was detected by inhibition of starch degradation. Flooding with iodine identified any remaining starch resulting in blue zones around producing colonies. The problems of this method were two-fold.

Firstly, it was relatively insensitive, the maximum sensitivity using paper discs soaked in test solutions of DNJ and placed on the agar surface was 1mg/ml. Secondly, there were problems with the reproducibility of the assay. To improve the sensitivity of the assay the ability of DNJ to inhibit glycosidases was examined in a liquid system rather than on an agar plate.

Trehalase (E.C. 3.2.1.28) is an α -glucosidase it is inhibited by DNJ (Scofield et al., 1986) and has been adopted for the estimation of DNJ in agar samples and culture broths in preliminary physiological experiments (Chapter 3). However, it is not practical to use this methodology to screen several thousand transformants. The technique involved three incubation steps and each sample had to be individually read in a spectrophotometer.

This resulted in a shift of emphasis to inhibition of α -glucosidase (E.C. 3.2.1.20) using microtitre plate screening technology. This was an adaptation of the trehalase liquid system but involved fewer steps. Two stages were involved; each well of the 96-well microtitre plates was initially used to grow individual cultures in each well. The culture supernatants were extracted and separated by filtration before the inhibition was measured. The ease of transfer of the supernatants allowed screening of a large number of transformants. The microtitre plate system fulfills two objectives; clones producing DNJ or NOJ can be detected within a library of several thousand colonies and titres of NOJ

and titres of NOJ and DNJ can be accurately assessed in culture broths.

5.2 Enzyme kinetics

The α -glucosidase enzyme assay used a substrate with a glycosidic linked nitrophenyl group which was liberated by α -glucosidase Fig 5.1 (Tilbeurgh et al., 1988). The reaction can be summarised by the general equation:



k_p = Catalytic constant rate $ES \longrightarrow E + P$

$k-1$ = $[E] [S]$

$k1$ = $[ES]$

The amount of product was measured by liberation of p-nitrophenol which was coloured yellow in solution.

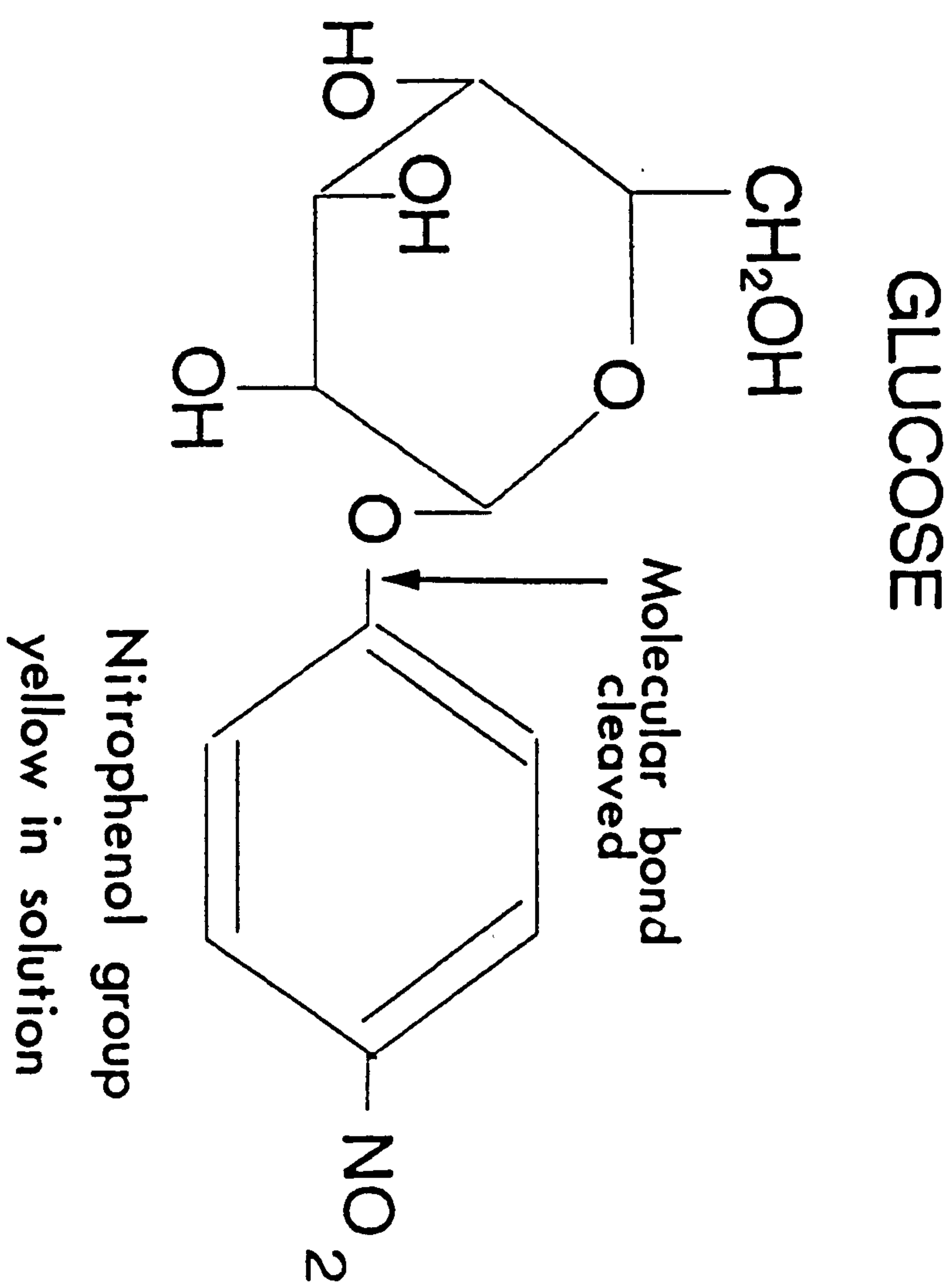
One of the primary objectives was to calculate the K_m of reactions involving α -glucosidase. The K_m is defined as the substrate concentration for half the V_{max} and is related to the general equation above.

$$K_m = \frac{k-1 + kp}{k1}$$

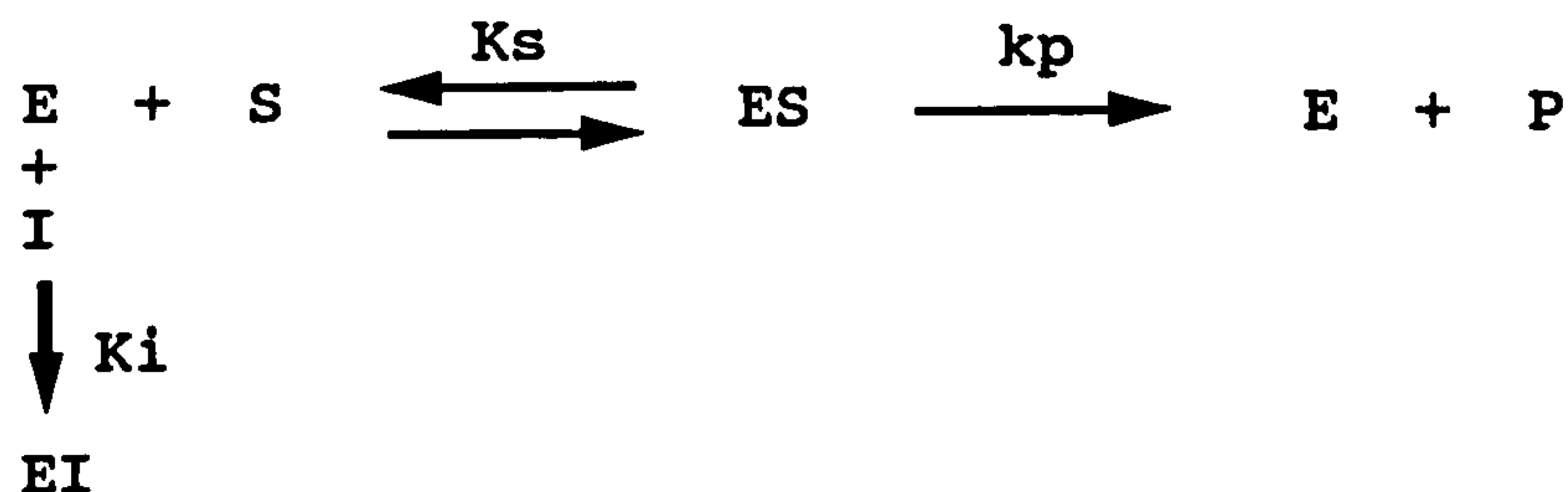
The K_m represents a valuable constant that related substrate concentration to the velocity of the enzyme catalysed reaction. At substrate concentrations less than

Fig 5.1

p-NITROPHENYL α-D-GLUCOPYRANOSIDE



twice the K_m , the reaction would not attain V_{max} . Calculation of the K_m allowed the development of a defined assay system. Excess substrate would be in competition with the inhibitor for the active site of the enzyme. Once the minimum substrate concentration was determined the concentration of enzyme was varied to determine a concentration that ensured that all the enzyme was used to make the enzyme-substrate complex. It was at this point that inhibitor can be introduced to examine the affect on reaction rate.



This reaction is reversible by addition of more substrate (to make more ES) and then initial velocity becomes equal to V_{max} . The apparent K_m can increase in the presence of a competitive inhibitor because at any concentration of inhibitor a proportion of enzyme exists as the enzyme-inhibitor complex, and has little affinity for the substrate. After the K_m and V_{max} have been calculated the K_i values for both DNJ and NOJ can be determined.

$$K_i = \frac{[E][I]}{[EI]}$$

The K_i is equal to the concentration of competitive inhibitor which apparently doubles the value of K_m . The

K_i is the inhibitor constant and indicates the affinity of the inhibitor for the active site of the enzyme.

5.3 RESULTS

5.3.1 ENZYME SUBSTRATE REACTIONS

5.2.3 Determination of V_{max} and K_m .

An α -glucosidase enzyme concentration of 0.025 of a unit was added to microtitre wells containing 4 different substrate concentrations. Initial velocity, which is a measure of reaction rate increased with increasing concentration of substrate. The calculated values for V_{max} and K_m for the assay (replicated 4 times) are shown below.

	V_{max} (a.u./min)	K_m (M)
Mean	0.242	2.0×10^{-5}
Std Err	0.018	0.27×10^{-5}

The mean K_m value from these results indicated that V_{max} would not be attained with a substrate concentration less than 4.0×10^{-5} M. The substrate concentrations used for the assay were above 4.0×10^{-5} M. so that the substrate concentration would not limit the reaction rate.

5.3.3 Effect of Enzyme Concentration

The aim of the experiment was to assess an appropriate amount of α -glucosidase for the assay. The reaction rates were linear and equal in Fig 5.2, when 0.1 and 0.5 units of enzyme were assayed showing the reaction rate was substrate limited. The reaction rate using 0.025 of a unit was lower, so all the enzyme would have made enzyme-substrate complex. The control line shows the absorbance of a reaction containing no enzyme. The only colour produced in this reaction is natural dissociation of substrate from the glucopyranoside molecule.

The enzyme concentration of 0.025 of a unit was selected for the assay because sensitivity to inhibitor is increased when no excess enzyme is present.

5.3.4 Deoxynojirimycin as an inhibitor of α -glucosidase

The effect of adding DNJ was observed by comparing the reaction rates between inhibited and uninhibited reactions. This is illustrated by the Lineweaver-Burke plot Fig 5.3. Analysis of the results of 4 uninhibited reactions and 16 reactions containing levels of DNJ and NOJ between 5×10^{-7} M. and 5×10^{-5} M., demonstrated NOJ and DNJ to be competitive inhibitors. The calculated values for V_{max} , K_m and K_i are illustrated in table 5.1.

Fig 5.2

THE RATES OF REACTION CAUSED BY DIFFERENT CONCENTRATIONS OF α -GLUCOSIDASE IN THE PRESENCE OF p-NITROPHENYL α -D-GLUCOPYRANOSIDE.

The substrate concentration was 9.2×10^{-5} M and 0.5, 0.1 and 0.025 units of enzyme were used in the reaction. The control sample contained no enzyme. The reactions were carried out at 37°C, pH 6.8, with the liberation of colour producing an absorbance which was assessed at 405nm. The reaction was replicated 3 times and the standard error was ($p=0.05$).

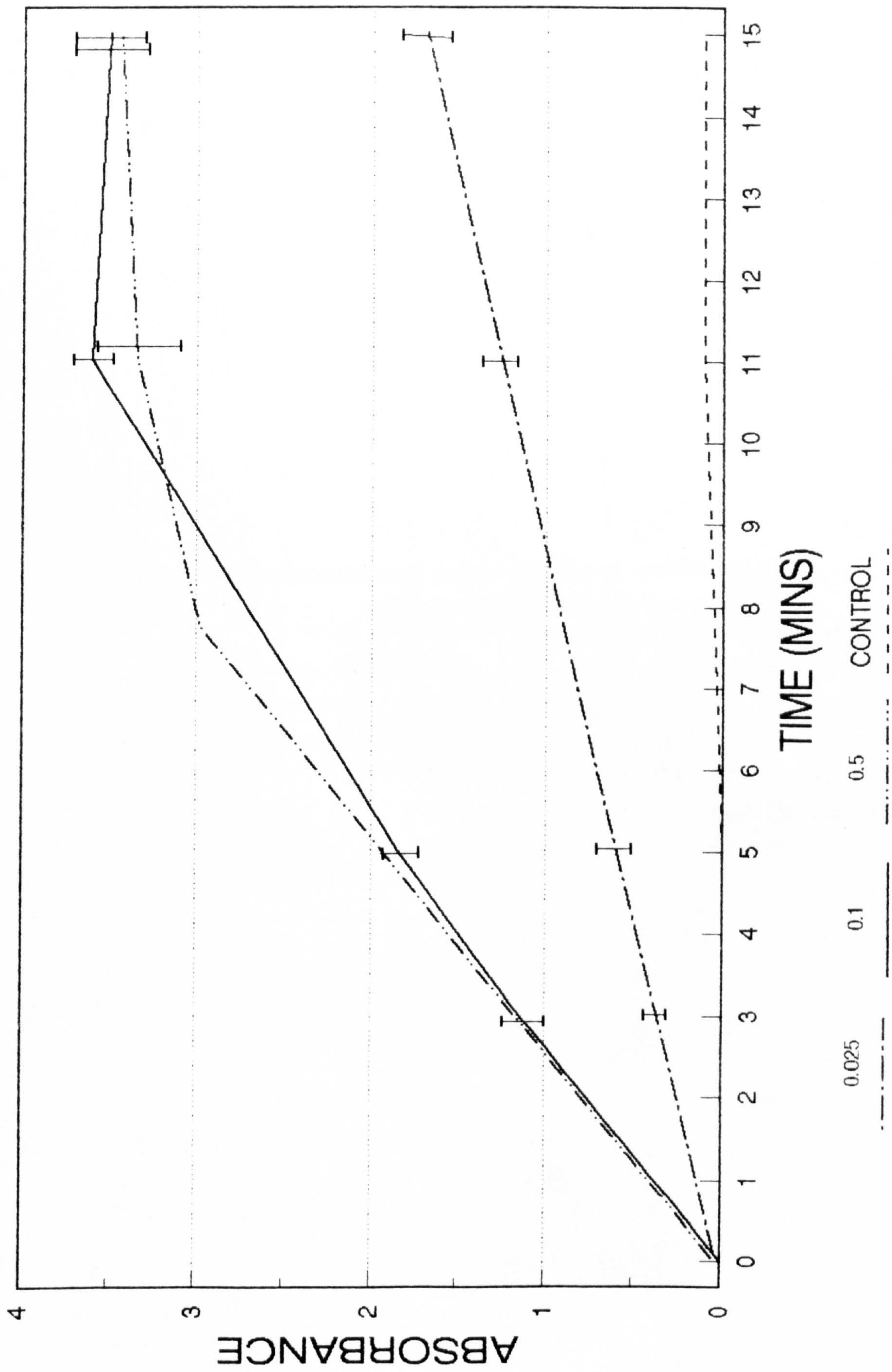
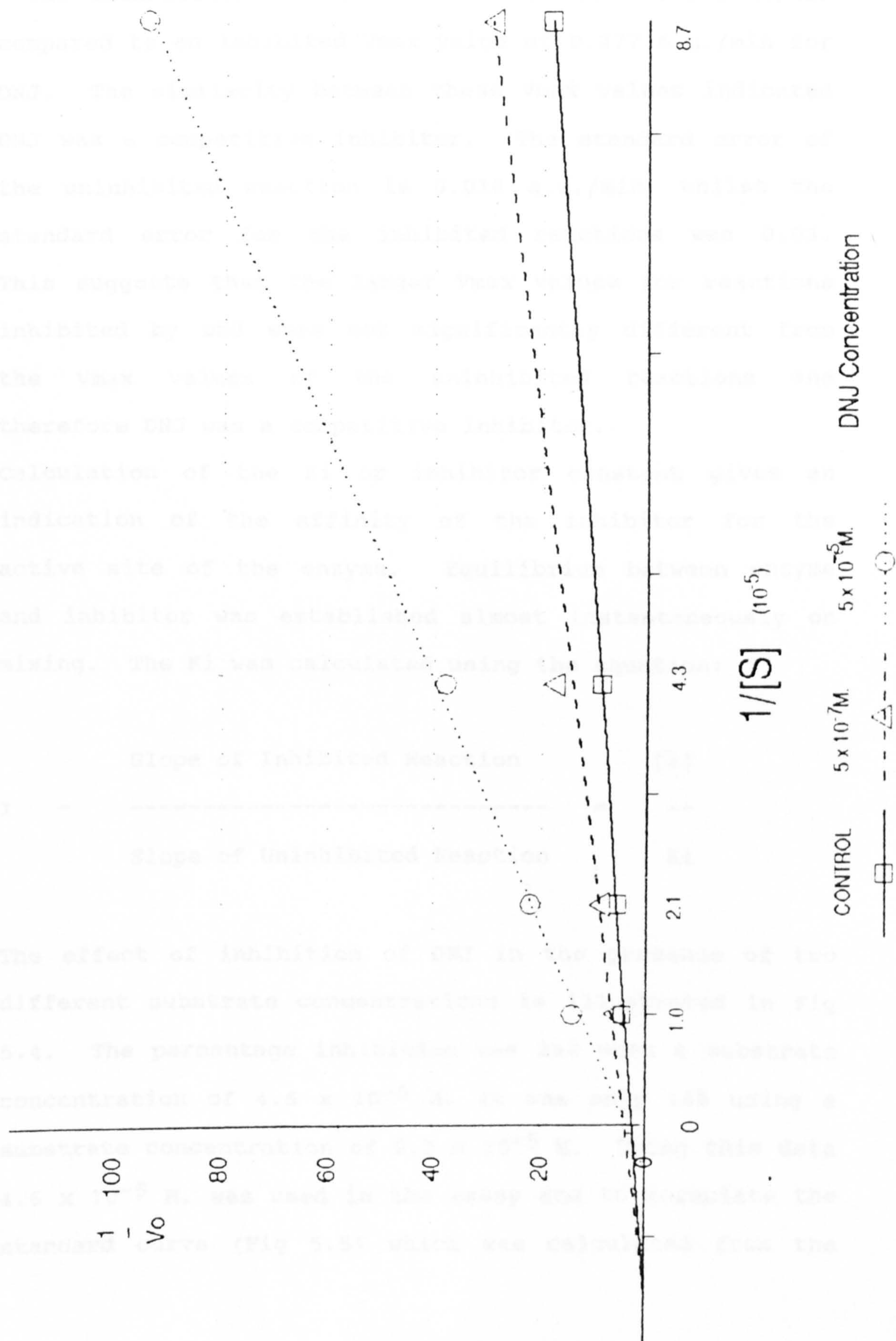


Fig 5.3

LINEWEAVER-BURKE PLOT OF THE INHIBITION OF
 α -GLUCOSIDASE IN THE PRESENCE OF DIFFERENT
CONCENTRATIONS OF DEOXYNOJIRIMYCIN.

All reactions at 37°C, pH 6.8 and absorbance 405nm.
The control rate contains no DNJ.



The uninhibited reaction had a V_{max} of 0.242 a.u./min compared to an inhibited V_{max} value of 0.277 a.u./min for DNJ. The similarity between these V_{max} values indicated DNJ was a competitive inhibitor. The standard error of the uninhibited reaction is 0.018 a.u./min, whilst the standard error for the inhibited reactions was 0.03. This suggests that the larger V_{max} values for reactions inhibited by DNJ were not significantly different from the V_{max} values of the uninhibited reactions and therefore DNJ was a competitive inhibitor.

Calculation of the K_i or inhibitor constant gives an indication of the affinity of the inhibitor for the active site of the enzyme. Equilibrium between enzyme and inhibitor was established almost instantaneously on mixing. The K_i was calculated using the equation:

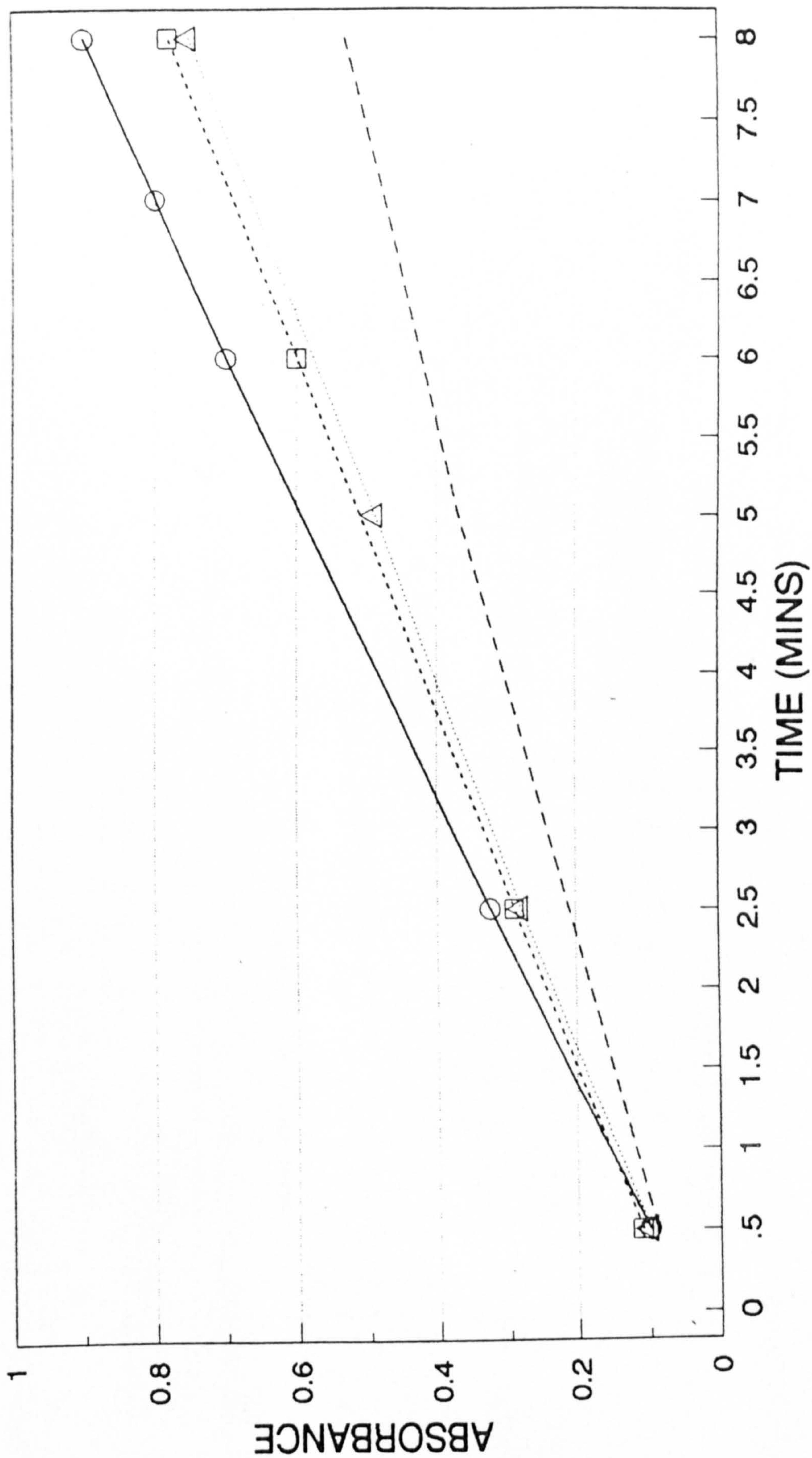
$$1 - \frac{\text{Slope of Inhibited Reaction}}{\text{Slope of Uninhibited Reaction}} = \frac{[I]}{K_i}$$

The effect of inhibition of DNJ in the presence of two different substrate concentrations is illustrated in Fig 5.4. The percentage inhibition was 29% when a substrate concentration of 4.6×10^{-5} M. It was only 16% using a substrate concentration of 9.2×10^{-5} M. Using this data 4.6×10^{-5} M. was used in the assay and to formulate the standard curve (Fig 5.5) which was calculated from the

Fig 5.4

THE EFFECT OF GLUCOPYRANOSIDE CONCENTRATION
ON α -GLUCOSIDASE ACTIVITY IN THE PRESENCE OF DNJ.

The reaction was carried out at 37°C, pH 6.8 and absorbance assessed at 405nm. The DNJ concentration of each reaction was 5×10^{-7} M. The substrate concentration in molarity is displayed below the graph and the concentration of α -glucosidase was set at 0.025 of a unit.



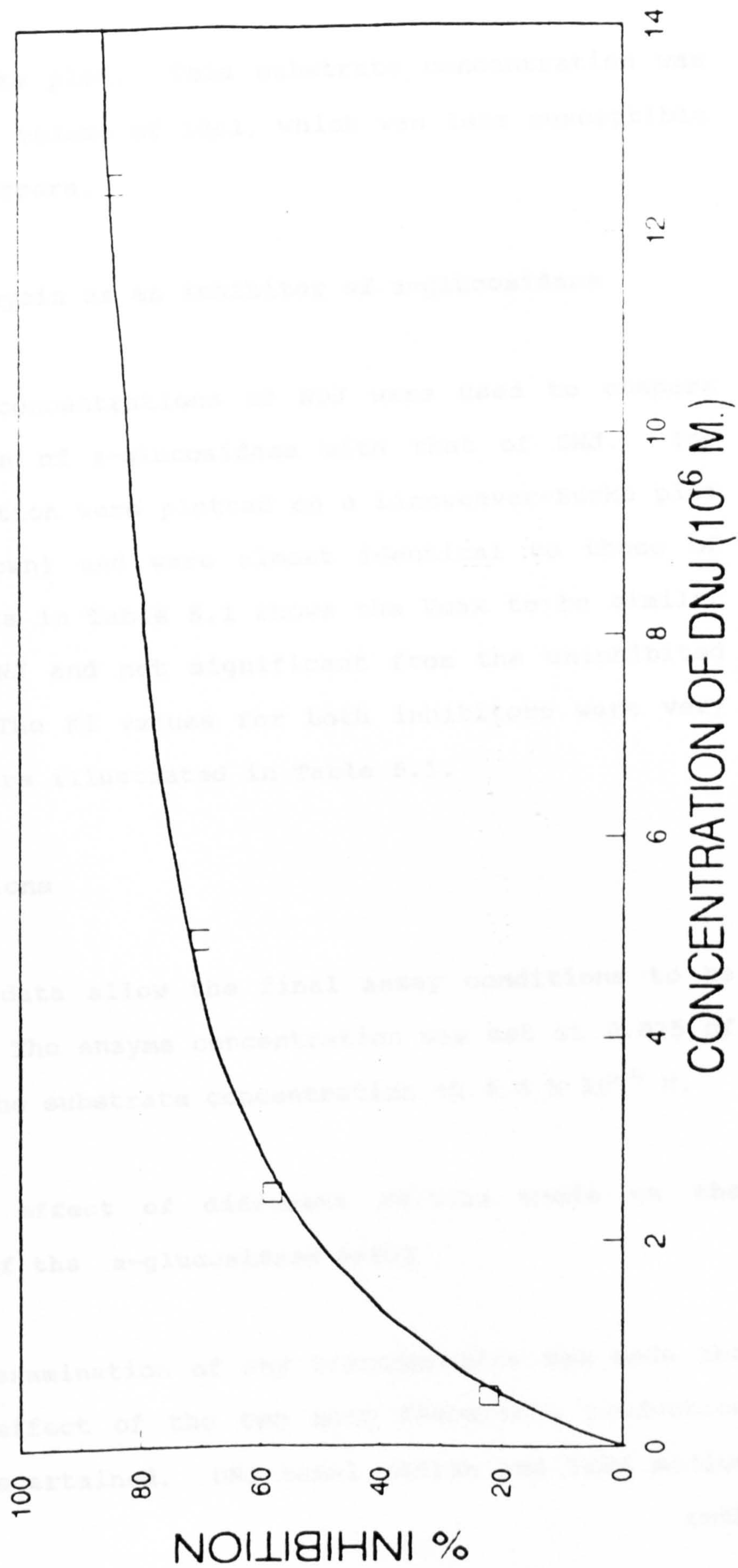
Substrate conc : $9.2 \times 10^{-5} M$ $4.6 \times 10^{-5} M$ $9.2 \times 10^{-6} M$ $4.6 \times 10^{-5} M$

No DNJ. No DNJ. DNJ ($5 \times 10^{-5} M$) DNJ ($5 \times 10^{-5} M$)

Fig 5.5

STANDARD CURVE SHOWING PERCENTAGE INHIBITION OF α -GLUCOSIDASE
AGAINST CONCENTRATION OF DEOXYNOJIRIMYCIN.

Concentration of DNJ in μ mols



Lineweaver-Burke plot. This substrate concentration was dispensed in a volume of 10 μ l, which was less susceptible to pipetting errors.

5.3.5 Nojirimycin as an inhibitor of α -glucosidase

Identical concentrations of NOJ were used to compare the inhibition of α -glucosidase with that of DNJ. The rates of reaction were plotted on a Lineweaver-Burke plot (data not shown) and were almost identical to those of DNJ. The data in Table 5.1 shows the V_{max} to be similar to that of DNJ and not significant from the uninhibited reaction. The K_i values for both inhibitors were very similar and are illustrated in Table 5.1.

Assay conditions

All these data allow the final assay conditions to be determined. The enzyme concentration was set at 0.025 of a unit and the substrate concentration at 4.6×10^{-5} M.

5.3.6 The effect of different culture media on the inhibition of the α -glucosidase assay.

Before the examination of any transformants was made the inhibitory effect of the two most favourable production media was ascertained. DNJ basal medium and ISP4 medium have both

Table 5.1

A COMPARISON OF THE ENZYME KINETICS OF
 α -GLUCOSIDASE IN THE PRESENCE AND ABSENCE
OF DEOXYNOJIRIMYCIN AND NOJIRIMYCIN.

All reactions at 37°C, pH 6.8 and absorbance assessed at 405nm.
The concentration of enzyme was 0.025 of a unit.

	α -D-GLUCOSIDASE UNINHIBITED	DEOXYNOJIRIMYCIN	NOJIRIMYCIN
Vmax (a.u./min)	0.242 SE 0.018	0.277 SE 0.03	0.261 SE 0.02
Km (M.)	2×10^{-5}	-	-
Ki (M.)	-	2.2×10^{-6}	2.4×10^{-6}
Km/Ki (ratio)	-	9.1	8.4

previously been used to produce DNJ. The amount of inhibition caused by the medium was 16% and 26% respectively. The inhibition detected may have been partially caused by starch molecules acting as an alternative substrate for α -glucosidase. The high background inhibition caused by ISP4 medium led to its rejection and the exclusive use of DNJ medium to grow the cultures of transformants.

5.3.7 Estimation of the quantity of DNJ and NOJ produced in different culture media by selected *Streptomyces* strains.

This experiment demonstrated the ability of the assay to detect DNJ/NOJ produced by *S.lavendulae* in shake flasks. The percentage inhibition of enzyme reaction rates for the different strains are illustrated in Table 5.2. The statistical significance was evaluated using an analysis of variance for the different reaction rates, using the Genstat statistical package (Lane et al., 1987).

5.3.8 The DNJ basal medium

The highest antibiotic titres were obtained using the DNJ basal medium. After 24 hours there was no inhibition in the presence of supernatant from *S.lavendulae*. However, on day 2 there was a dramatic increase in the amount of inhibition from *S.lavendulae* to 96%. The other three samples only cause between 4% and 18%

Table 5.2

INHIBITION CAUSED BY SUPERNATANTS FROM
SELECTED Streptomyces STRAINS AGAINST
 α -GLUCOSIDASE.

Figures are % inhibition using DNJ basal medium against a water blank.

nd = No data.

* = Significant ($p=0.01$).

The substrate concentration was 4.6×10^{-5} M. and the enzyme concentration was 0.025 of a unit.

	FERMENTATION TIME IN DAYS	BLANK MEDIUM CONTROL	<u>S.LIVIDANS</u> TK24	<u>S.LAVENDULAE</u> 31434	<u>S.SUBRUTILUS</u> 445
	1	6	6	0	1
	2	18	11	96 *	4
	3	11	0	90 *	17
	4	5	1	88 *	1
	5	4	6	90 *	10
	6	23	0	92 *	32
	7	nd	nd	nd	nd
	8	17	0	91 *	40
	9	27	0	80 *	42
	10	34	0	97 *	51

inhibition. The level of inhibition from *S.lavendulae* was maintained throughout the experiment, with *S.lavendulae* significantly inhibiting the enzyme reaction at the 1% level. The production of DNJ/NOJ over 10 days is presented in Fig 5.6. *S.subbrutilus* produced no inhibitory effect until day 6 when the supernatant caused 32% inhibition, compared to 23% and 0% for the medium control and non-producing strain respectively. The controls for this experiment consisted of a water blank, uninoculated medium and supernatants of *S.lividans*. There was no consistent evidence of any inhibition produced from any of these controls throughout the experiment.

5.3.9 ISP4 medium

S.lavendulae only produced a small titre of DNJ (more than 10 times less) when cultured in the ISP4 medium (data not presented). This compounded with the larger background inhibition measured when using this medium led to DNJ basal medium being used exclusively in the development of the microtitre screening system.

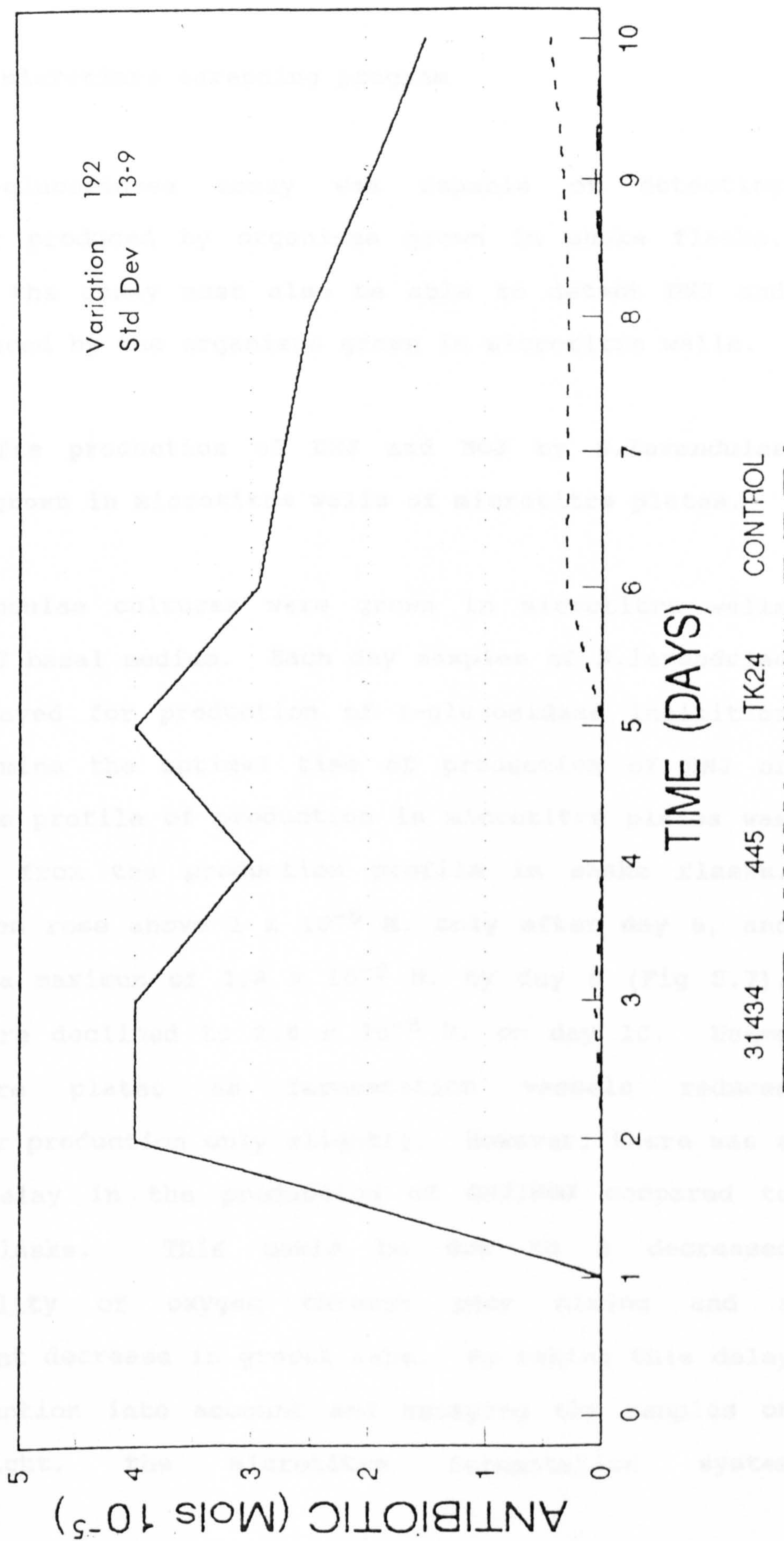
Fig 5-6

DEOXYNOJIRIMYCIN AND NOJIRIMYCIN PRODUCTION BY SELECTED *Streptomyces* STRAINS.

The figures were derived from continuous sampling of two replicates of each strain grown in DNJ basal medium.

The shake flasks were incubated at 28°C.

The substrate concentration was 4.6×10^{-5} M. and the enzyme concentration was 0.025 of a unit.



5.4 The microtitre screening program

The α -glucosidase assay was capable of detecting inhibitor produced by organisms grown in shake flasks. However, the assay must also be able to detect DNJ and NOJ produced by the organisms grown in microtitre wells.

5.4.1 The production of DNJ and NOJ by *S.lavendulae* (31434) grown in microtitre wells of microtitre plates.

S.lavendulae cultures were grown in microtitre wells using DNJ basal medium. Each day samples of *S.lavendulae* were assayed for production of α -glucosidase inhibition to determine the optimal time of production of DNJ or NOJ. The profile of production in microtitre plates was distinct from the production profile in shake flasks. Production rose above 1×10^{-5} M. only after day 6, and reached a maximum of 3.8×10^{-5} M. by day 8 (Fig 5.7). This titre declined to 2.8×10^{-5} M. on day 10. Using microtitre plates as fermentation vessels reduced inhibitor production only slightly. However, there was a 5 day delay in the production of DNJ/NOJ compared to shake flasks. This could be due to a decreased availability of oxygen through poor mixing and a consequent decrease in growth rate. By taking this delay in production into account and assaying the samples on day eight, the microtitre fermentation system

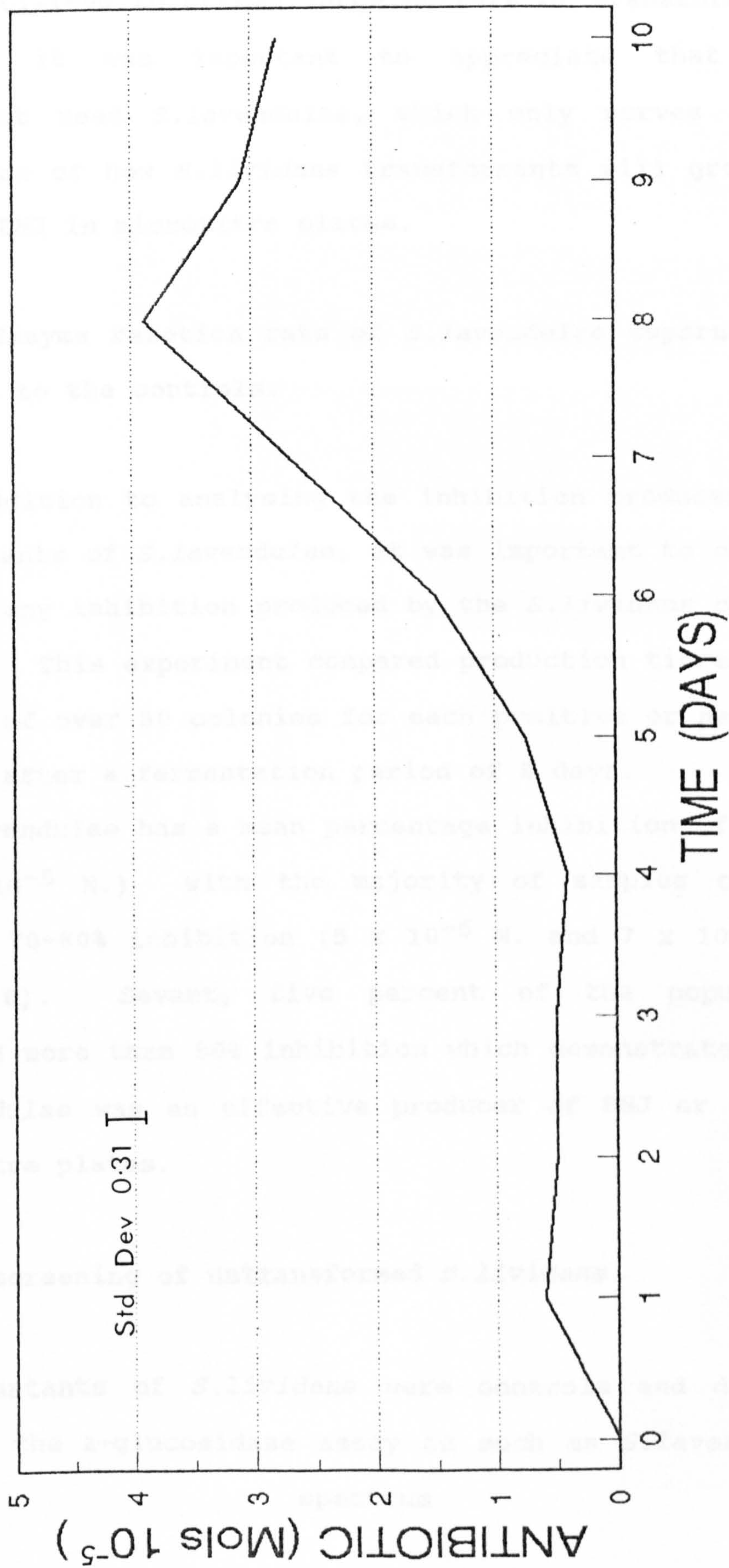
Fig 5.7

DEOXYNOJIRIMYCIN AND NOJIRIMYCIN PRODUCTION
BY *S.lavendulae* (31434) IN MICROTITRE PLATES

MICROTITRES INCUBATED AT 28°C

FIGURES DERIVED FROM THE MEAN OF TWELVE SAMPLES.

The substrate concentration was 4.6×10^{-5} M. and the enzyme concentration was set at 0.025 of a unit.



31434

can be utilised to screen large numbers of transformants. However, it was important to appreciate that this experiment used *S.lavendulae*, which only serves as an indication of how *S.lividans* transformants will grow and produce DNJ in microtitre plates.

5.4.2 Enzyme reaction rate of *S.lavendulae* supernatants compared to the controls.

In addition to analysing the inhibition produced from supernatants of *S.lavendulae*, it was important to compare this to any inhibition produced by the *S.lividans* control samples. This experiment compared production titres from samples of over 80 colonies for each positive or negative control after a fermentation period of 8 days.

S.lavendulae has a mean percentage inhibition of 67% (3.5×10^{-5} M.) with the majority of samples causing between 70-80% inhibition (5×10^{-5} M. and 7×10^{-5} M.) (Fig 5.8). Seventy five percent of the population produced more than 50% inhibition which demonstrated that *S.lavendulae* was an effective producer of DNJ or NOJ in microtitre plates.

5.4.3 Screening of untransformed *S.lividans*.

Supernatants of *S.lividans* were controls and did not inhibit the α -glucosidase assay as much as *S.lavendulae*.
The spectrum of

Fig 5.8

THE SCREENING OF THE SUPERNATANTS OF *S.lavendulae*.

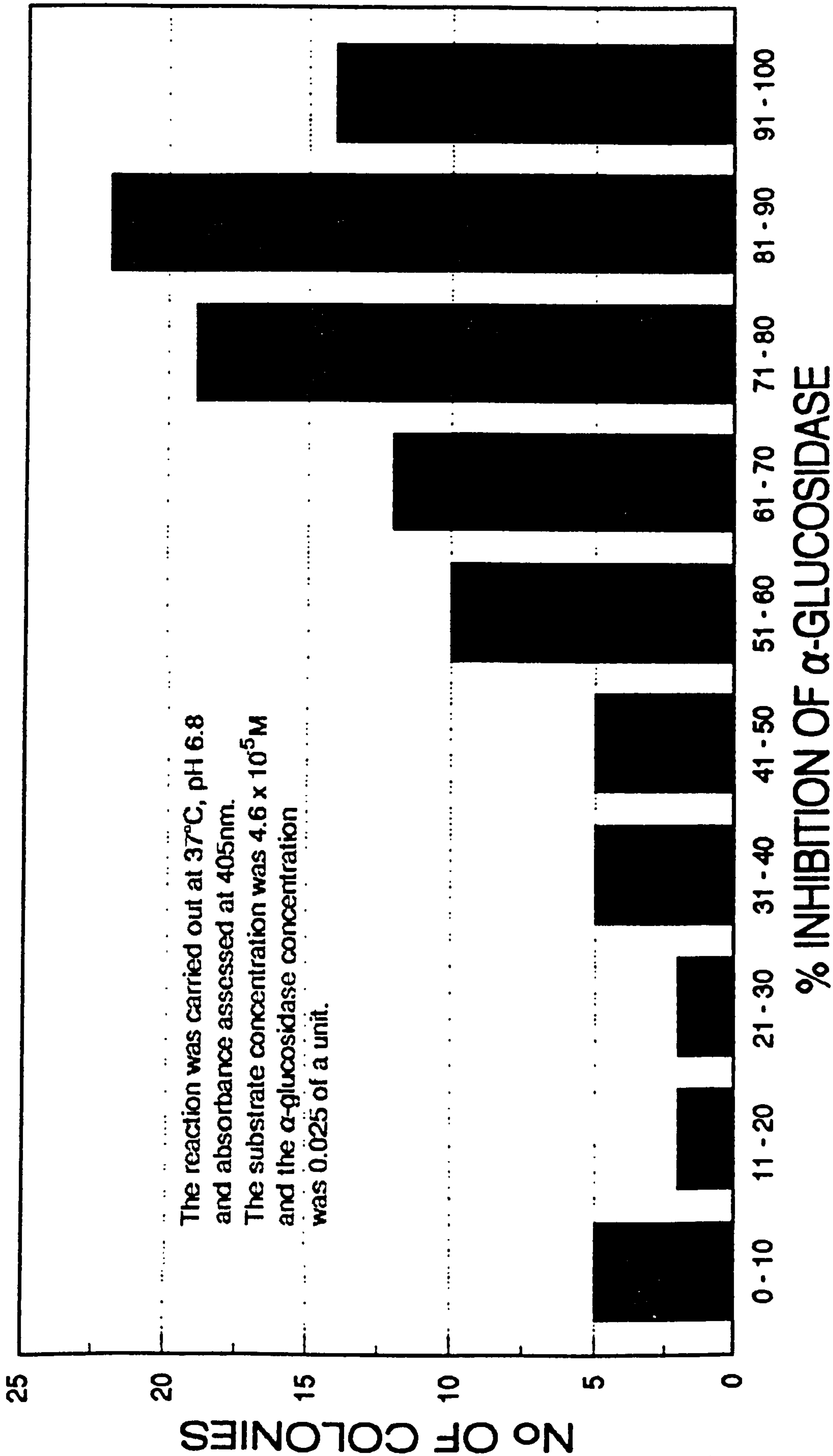
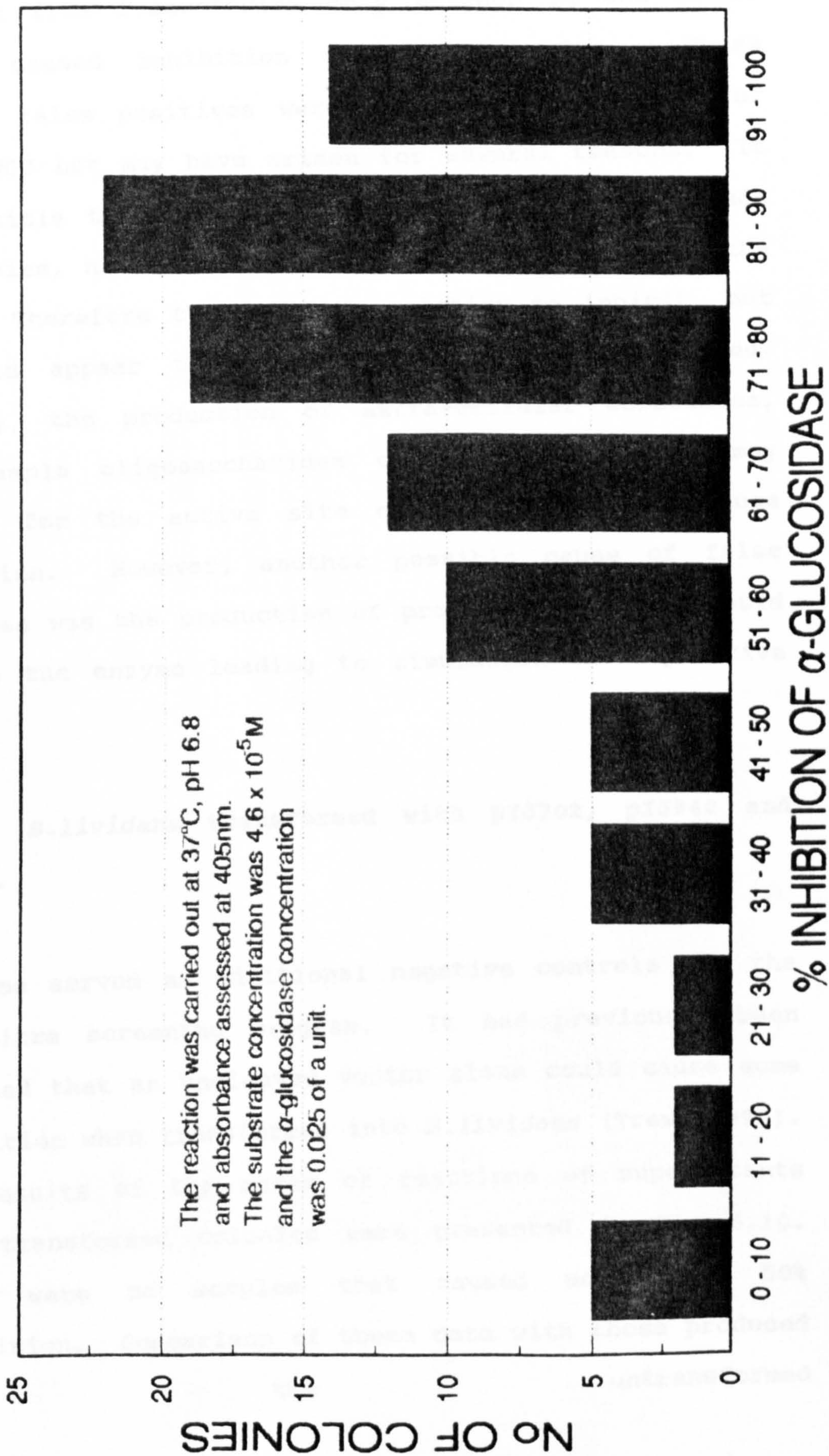


Fig 5.8

THE SCREENING OF THE SUPERNATANTS OF *S.lavendulae*.



inhibition for *S.lividans* illustrated in Fig 5.9 was very different from *S.lavendulae* only between 1% and 2% of samples caused inhibition greater than 50%. These apparent false positives were not due to production of DNJ or NOJ but may have arisen for several reasons. It was possible that during the screening of many hundreds of colonies, no enzyme entered a small proportion of the wells. Therefore there was no reaction to inhibit, but it would appear the reaction was totally inhibited. Secondly, the production of extra-cellular substances, for example oligosaccharides or starch dextrans, may compete for the active site of the enzyme and cause inhibition. However, another possible cause of false positives was the production of proteases as these would degrade the enzyme leading to simulation of a positive result.

5.4.4 *S.lividans* transformed with pIJ702, pIJ940 and pIJ916.

These served as additional negative controls for the microtitre screening program. It had previously been reported that an uncleaved vector alone could cause some inhibition when transformed into *S.lividans* (Trew, 1992). The results of the rates of reactions of supernatants from transformed colonies were presented in Fig 5.10. There were no samples that caused more than 50% inhibition. Comparison of these data with those produced from the untransformed

Fig 5-9
THE SCREENING OF UNTRANSFORMED *S.lividans*.

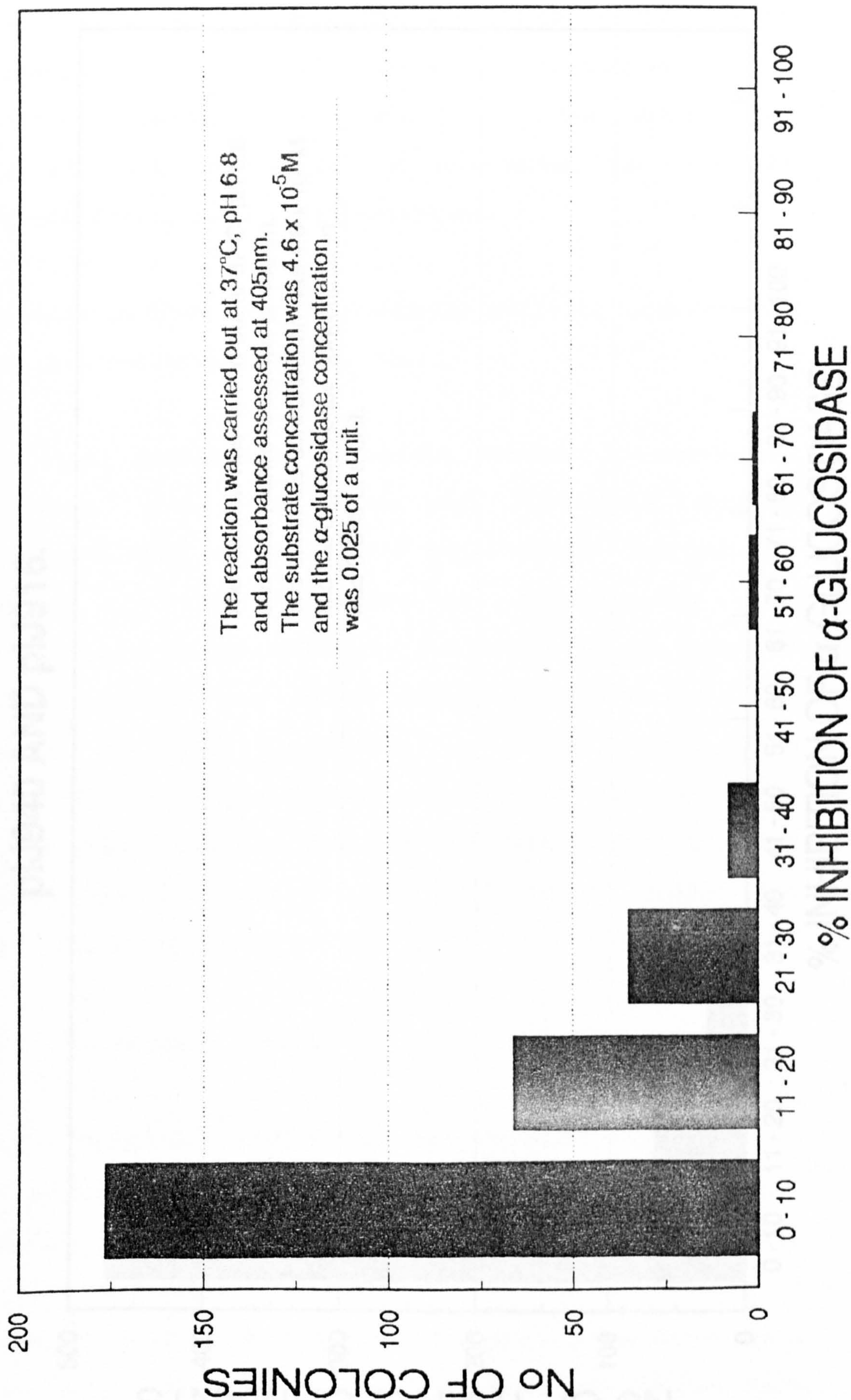
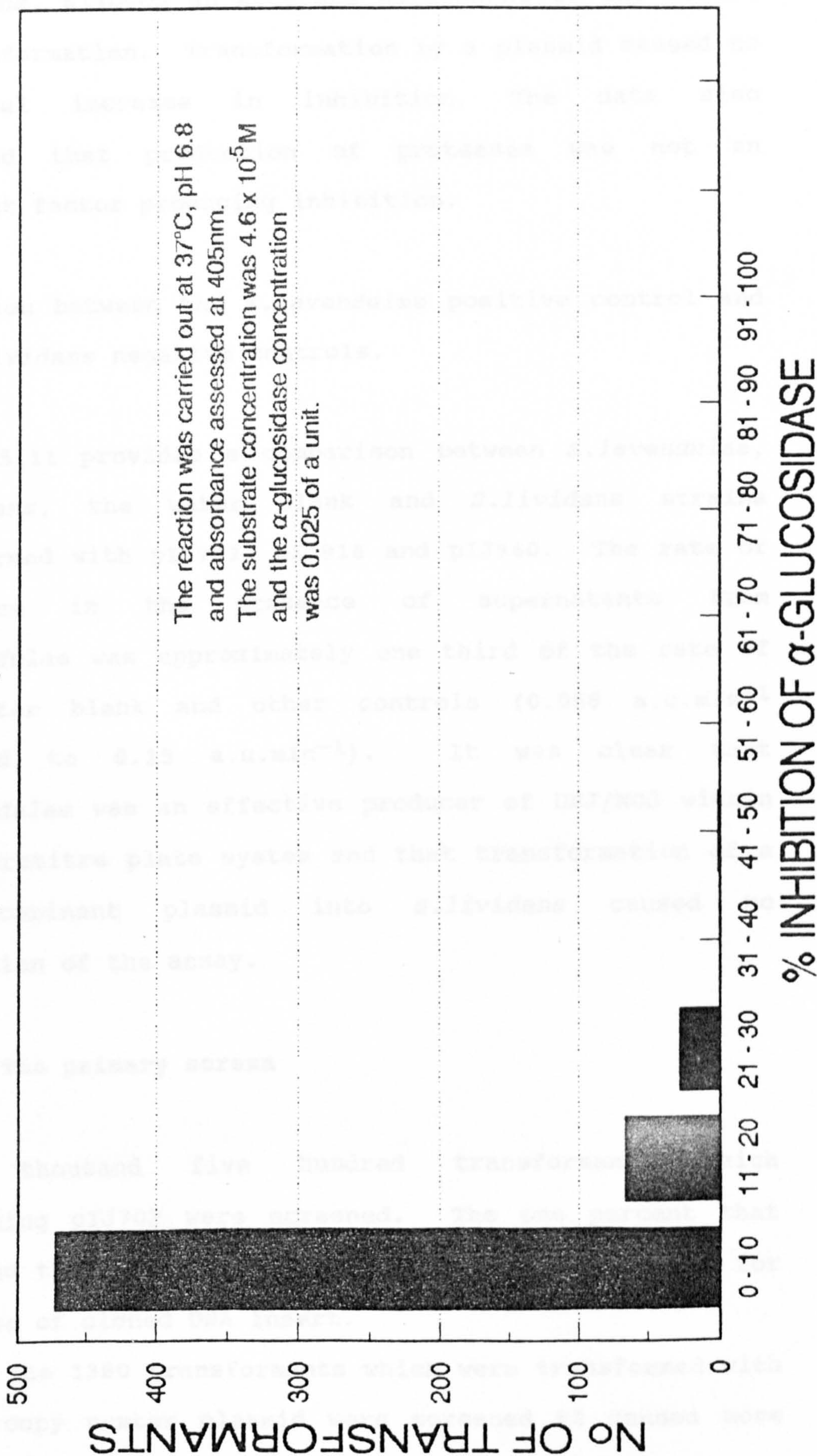


Fig 5.10
**SCREENING S.lividans TRANSFORMED WITH pIJ702,
 pIJ940 AND pIJ916.**



S.lividans, allowed an accurate assessment of the effect of transformation. Transformation by a plasmid caused no additional increase in inhibition. The data also indicated that production of proteases was not an important factor producing inhibition.

Comparison between the *S.lavendulae* positive control and the *S.lividans* negative controls.

Fig 5.11 provides a comparison between *S.lavendulae*, *S.lividans*, the water blank and *S.lividans* strains transformed with pIJ702, pIJ916 and pIJ940. The rate of reactions in the presence of supernatants from *S.lavendulae* was approximately one third of the rate of the water blank and other controls ($0.058 \text{ a.u.min}^{-1}$ compared to $0.15 \text{ a.u.min}^{-1}$). It was clear that *S.lavendulae* was an effective producer of DNJ/NOJ within the microtitre plate system and that transformation of a non-recombinant plasmid into *S.lividans* caused no inhibition of the assay.

5.4.5 The primary screen

Two thousand five hundred transformants which containing pIJ702 were screened. The one percent that produced the largest inhibition were further examined for the size of cloned DNA insert.

When the 1380 transformants which were transformed with a low copy number plasmid were screened 8% caused more

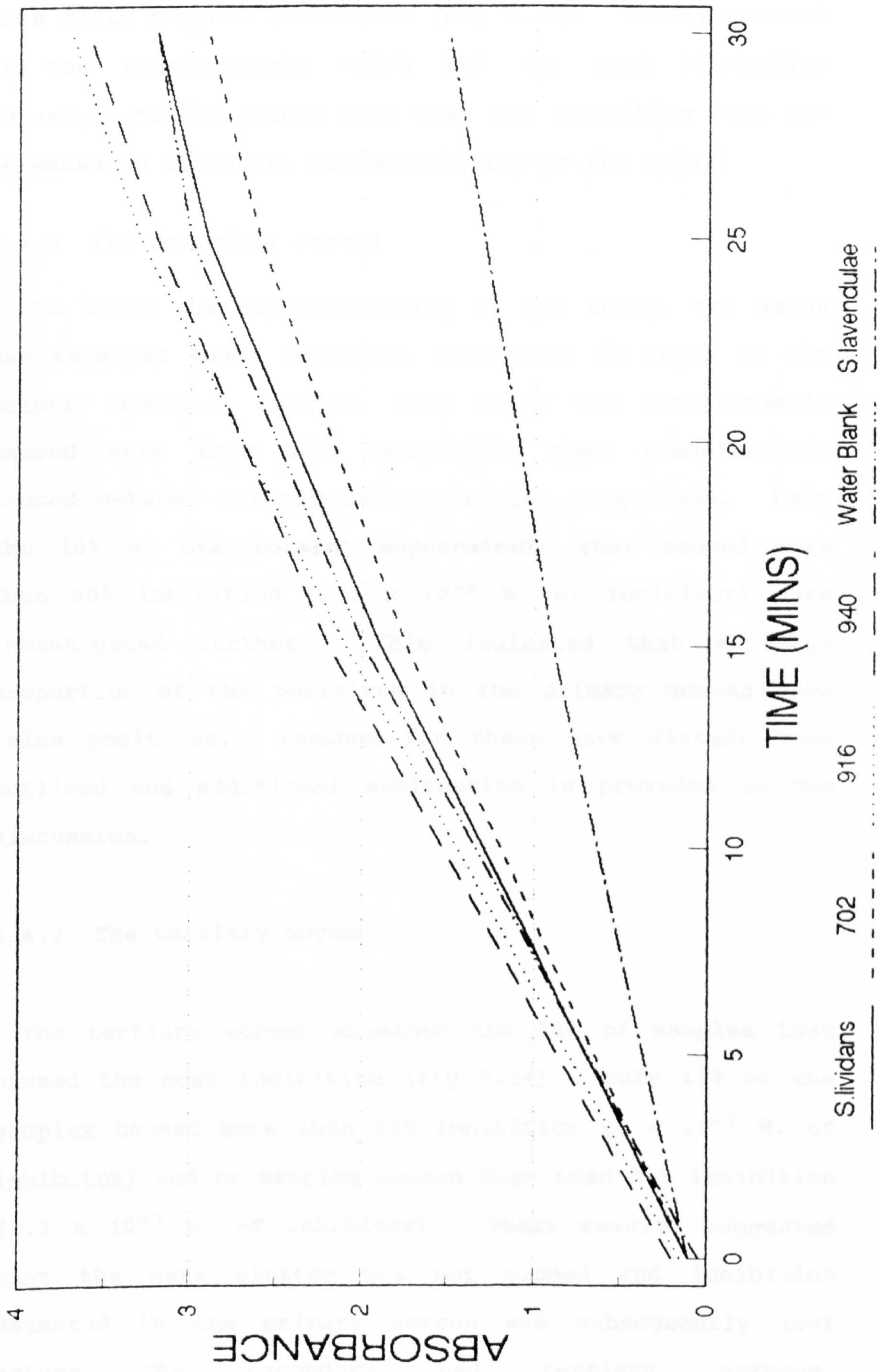
Fig 5.11

THE INHIBITION OF α -GLUCOSIDASE BY SUPERNATANTS
OF SELECTED *Streptomyces* STRAINS.

S.lividans - Untransformed *S.lividans*
S.lavendulae - Untransformed *S.lavendulae*
702 - *S.lividans* transformed with pIJ702
916 - *S.lividans* transformed with pIJ916
940 - *S.lividans* transformed with pIJ940
Water Blank - Distilled water

The reaction temperature was 37°C, pH 6.8.

The substrate concentration was 4.6×10^{-5} M. and the enzyme concentration was 0.025 of a unit.



than fifty percent inhibition (Fig 5.12). Twenty percent of the transformants which had the most bio-active supernatants and caused more than 40% inhibition were re-screened to check the reproducibility of the assay.

5.4.6 The secondary screen

To check the reproducibility of the assay, the assay was repeated under identical conditions to those of the primary screen. However, only 2% of the transformants caused more than 50% inhibition. Most transformants caused between 20% and 30% inhibition (Fig 5.13). Only the 15% of transformant supernatants that caused more than 40% inhibition (1.1×10^{-6} M. of inhibitor) were investigated further. This indicated that a large proportion of the positives in the primary screen were false positives, reasons for these have already been outlined and additional explanation is provided in the discussion.

5.4.7 The tertiary screen

The tertiary screen examined the 15% of samples that caused the most inhibition (Fig 5.14). Only 11% of the samples caused more than 20% inhibition (4×10^{-7} M. of inhibitor) and no samples caused more than 30% inhibition (6.3×10^{-7} M. of inhibitor). These results suggested that the gene cluster was not cloned and inhibition detected in the primary screen was subsequently lost before the secondary and tertiary screens.

Fig 5.12

THE PRIMARY SCREENING OF S.lividans TRANSFORMANTS.

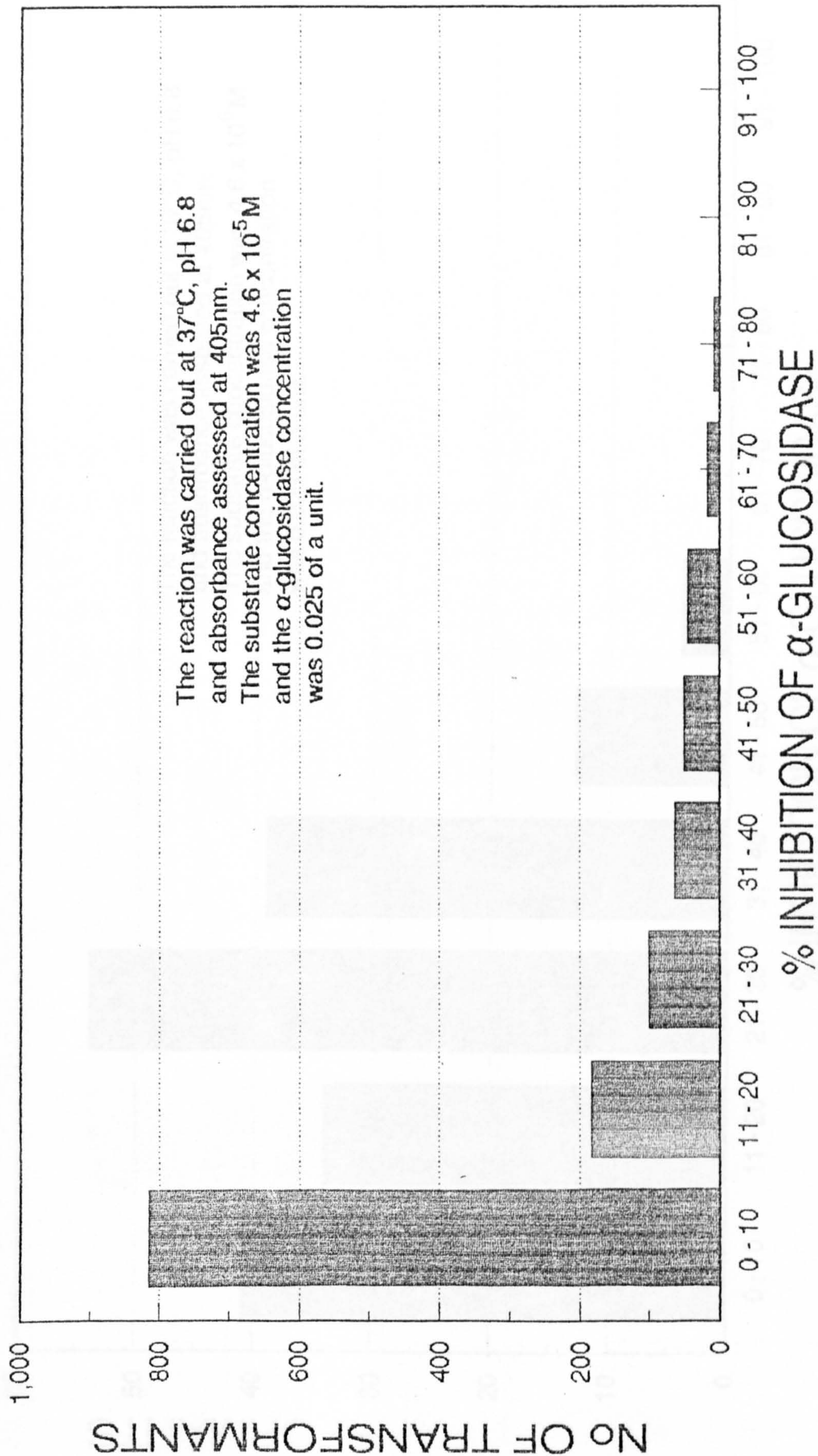


Fig 5.13

THE SECONDARY SCREENING OF S.lividans pIJ940 AND pIJ916 TRANSFORMANTS.

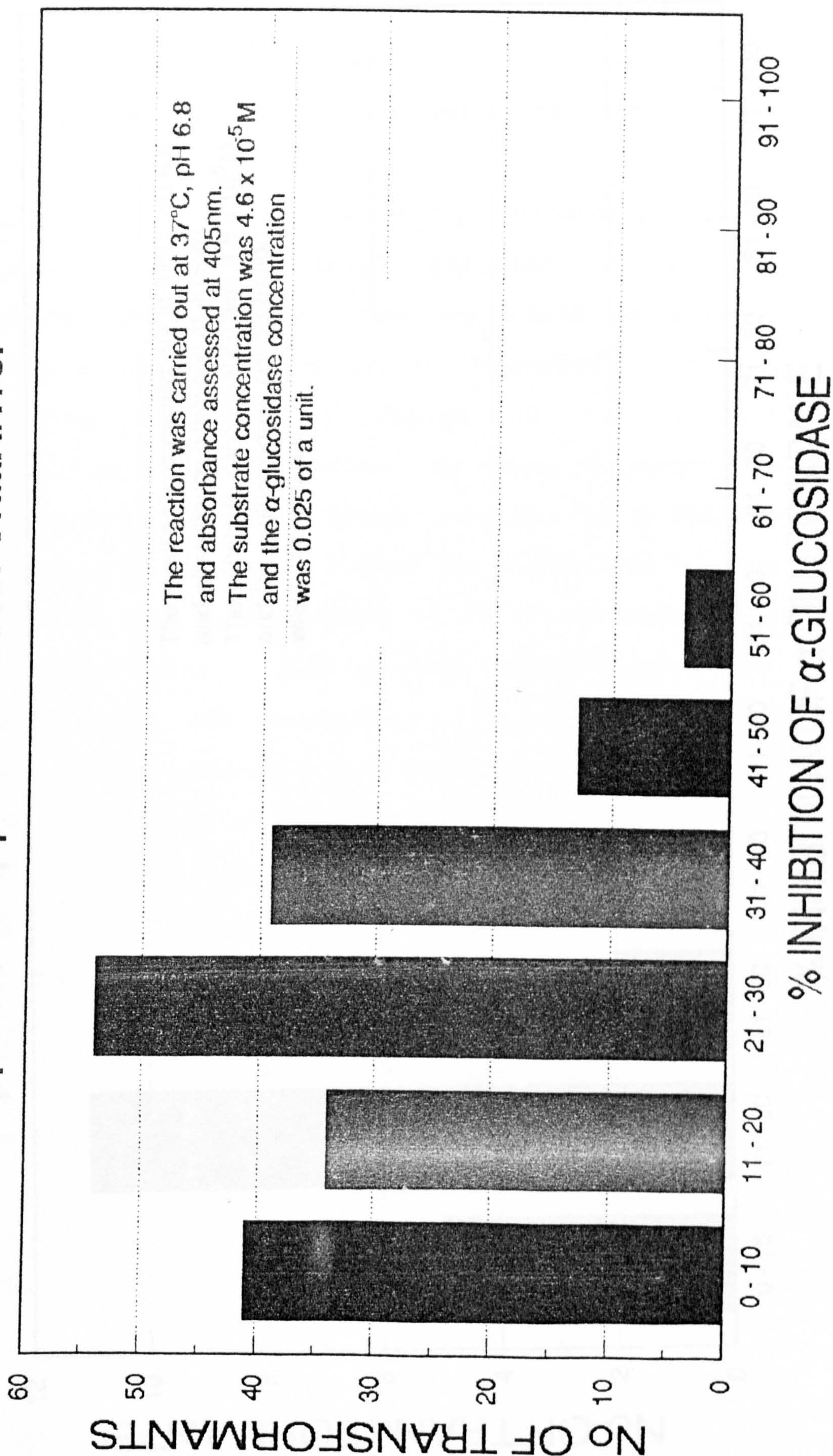
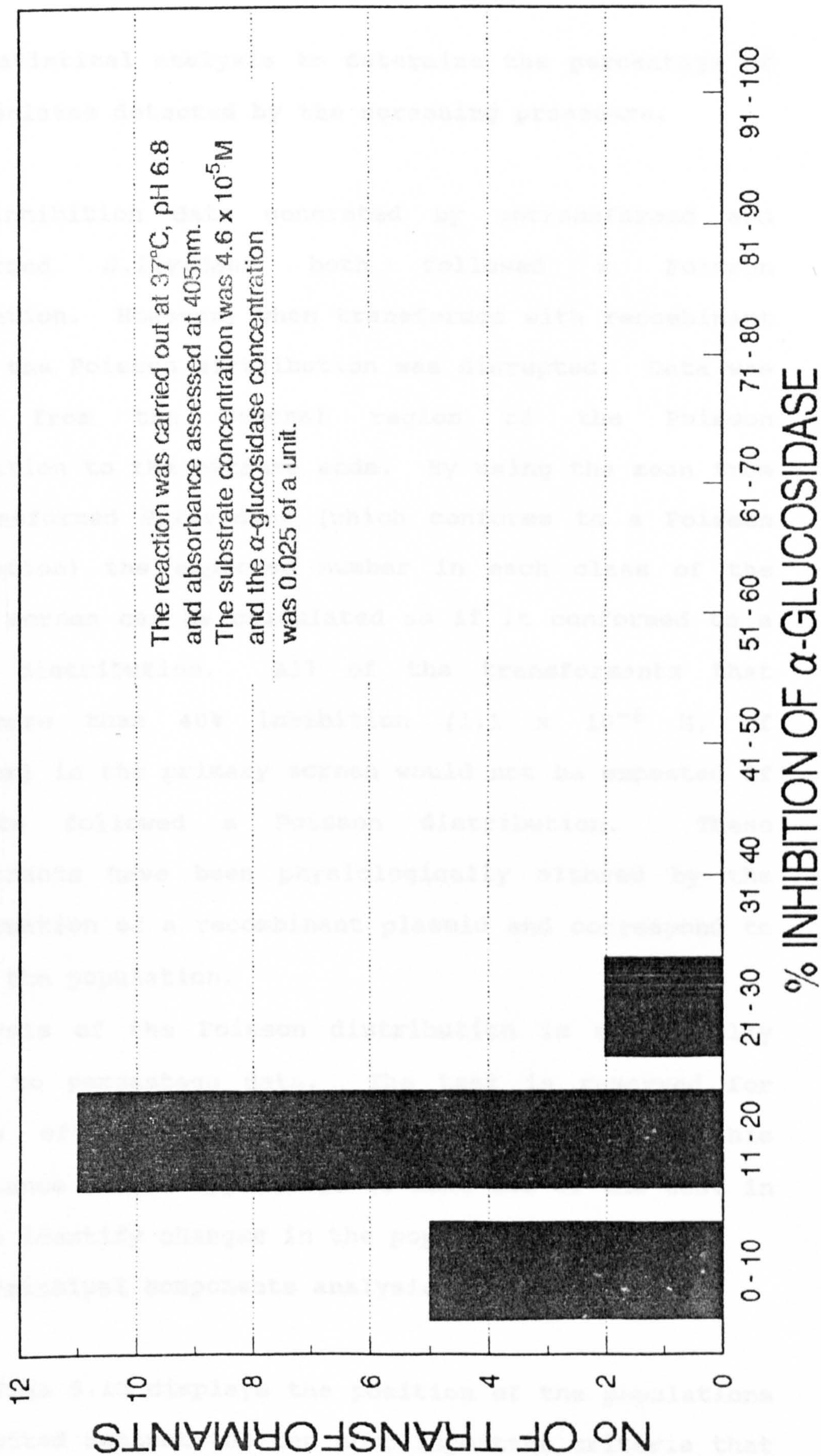


Fig 5.14

THE TERTIARY SCREENING OF *S.lividans* pIJ940 AND pIJ916 TRANSFORMANTS.



5.5 Statistical analysis to determine the percentage of novel isolates detected by the screening procedure.

The inhibition data generated by untransformed and transformed *S.lividans* both followed a Poisson distribution. However, when transformed with recombinant plasmid the Poisson distribution was disrupted. Data was shifted from the central region of the Poisson distribution to the extreme ends. By using the mean from the transformed *S.lividans* (which conforms to a Poisson distribution) the expected number in each class of the primary screen can be calculated as if it conformed to a Poisson distribution. All of the transformants that cause more than 40% inhibition (1.1×10^{-6} M. of inhibitor) in the primary screen would not be expected if the data followed a Poisson distribution. These transformants have been physiologically altered by the transformation of a recombinant plasmid and correspond to 8.2% of the population.

Analysis of the Poisson distribution is not usually applied to percentage data. The test is reserved for analysis of discontinuous data. However, in this circumstance it was applicable to make use of the test in order to identify changes in the population.

5.5.1 Principal components analysis

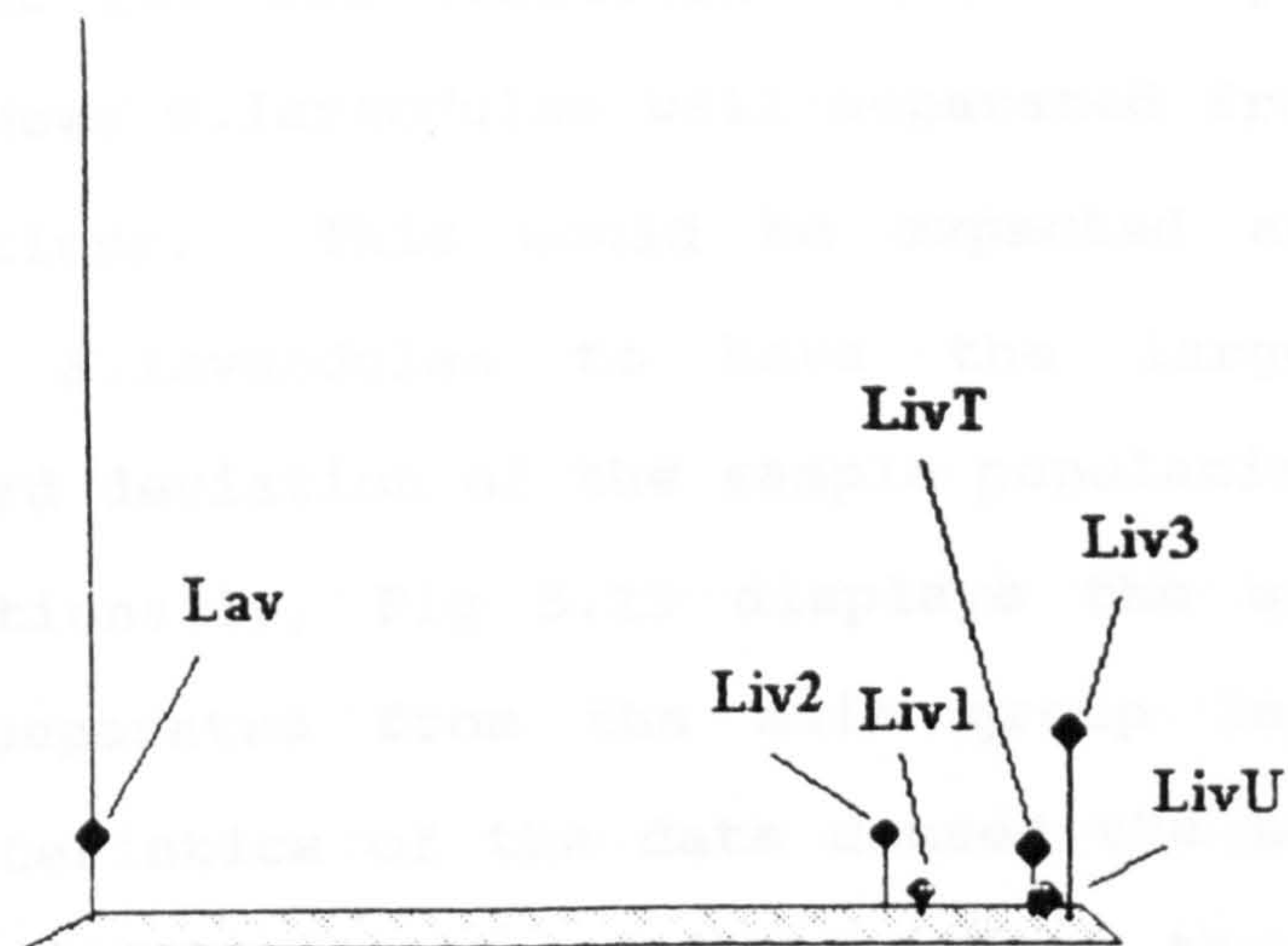
The Figs 5.15 displays the position of the populations when plotted against the ten most important criteria that

Fig 5-15

PRINCIPAL COMPONENTS ANALYSIS ON THE
SCREENED Streptomyces STRAINS.

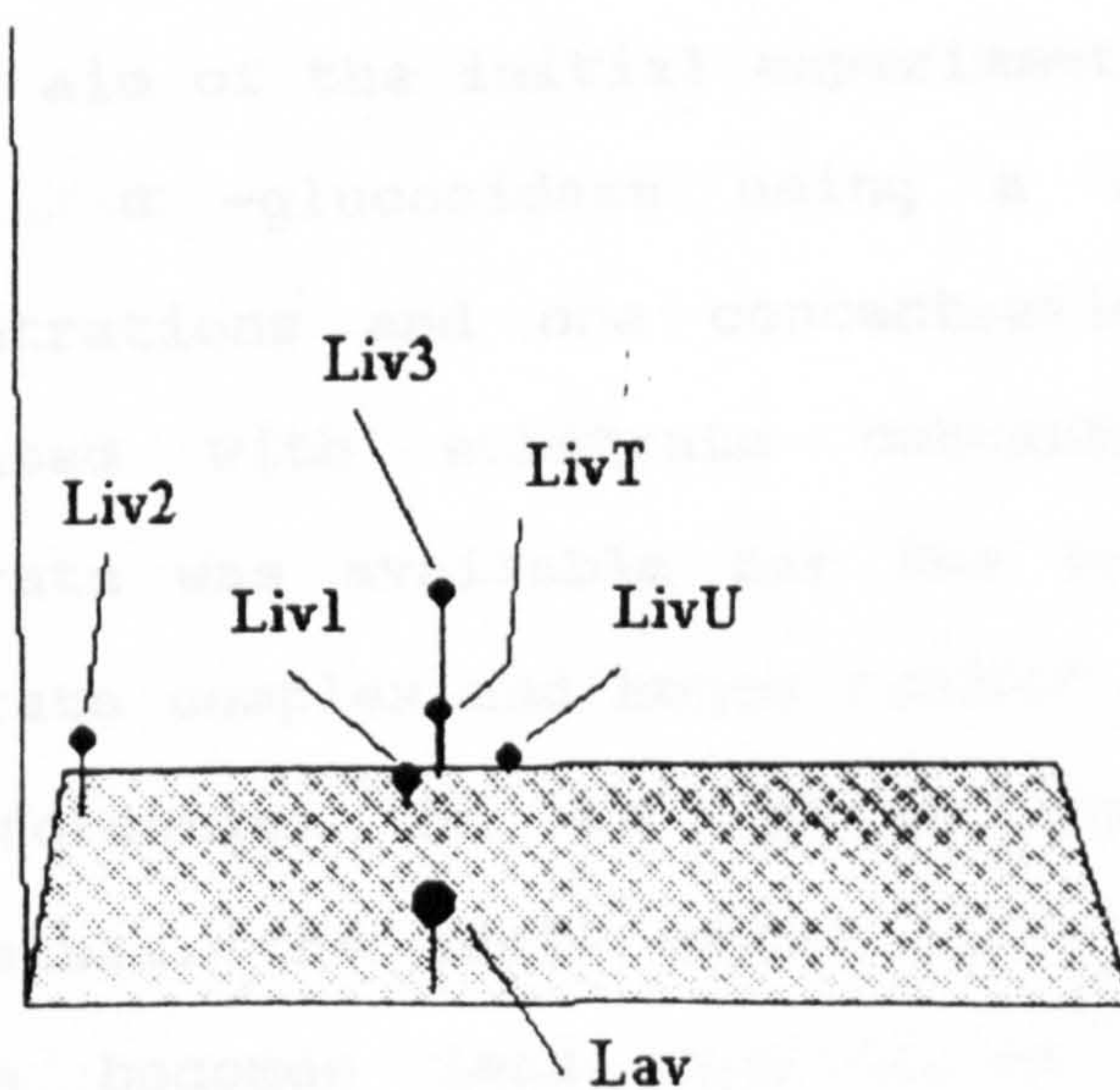
Lav = S.lavendulae
LivU = Untransformed S.lividans
LivT = Transformed S.lividans
Liv1 = Primary Screen
Liv2 = Secondary Screen
Liv3 = Tertiary Screen

The first diagram shows the S.lavendulae data separate from the other populations. However, when viewed from a different perspective the data comprising the secondary screening is also distinct from the other populations of data. See text for details.



2.6. Discussion

2.6.1 Asymptotic kinetics



account for the variation within the population. Fig 5.15 shows *S.lavendulae* well separated from all the other populations. This would be expected as previous data showed *S.lavendulae* to have the largest mean and standard deviation of the sample populations.

Additionally, Fig 5.15 displays the secondary screen, well separated from the main group in another plane. Characteristics of the data caused the separation of the data of the secondary screen from the primary screen. Yet the characteristics of the tertiary screen were similar to the primary screen and caused these two data sets to be clustered together.

5.6. Discussion

5.6.1 Enzyme kinetics

The aim of the initial experiment was to determine the K_m of α -glucosidase using a variety of substrate concentrations and one concentration of enzyme. V_{max} increased with substrate concentration because more substrate was available for the enzyme to form enzyme-substrate complex and hence product. Additionally, since the formation of an enzyme substrate complex was reversible, the small amount of substrate bound by the enzyme becomes less significant at higher substrate concentrations. The K_m for α -glucosidase was established to be 2.0×10^{-5} M., as a generalisation most K_m values lie in the range 10^{-2} - 10^{-6} M. (Palmer 1981). By using

a substrate concentration of twice the K_m , all the enzyme was utilised to form the enzyme-substrate complex and product was being made at the maximum rate.

The enzyme concentration was determined by assaying various enzyme concentrations against a substrate concentration greater than twice the K_m . When the enzyme was present in excess, it reached a maximal reaction rate limited by substrate concentration. At lower concentrations of enzyme, for example 0.025 of a unit, the substrate was not limiting and all the enzyme was used to form the enzyme-substrate complex. Once the substrate and enzyme concentrations had been fixed DNJ and NOJ were added to determine their effectiveness as inhibitors. Analysis of the initial rates of reaction and the effect of any inhibitors on the initial reaction rates was the most sensitive measure of inhibition. The rates of reaction were illustrated in a Lineweaver-Burke plot which additionally allowed values for V_{max} and K_m to be derived.

The sensitivity of the assay system for DNJ can also be determined from the graph. No inhibition was found using a DNJ final concentration of 2.5×10^{-7} M. There was also little difference in the inhibition profiles when DNJ concentrations were above 1×10^{-5} M. This was the maximum level of inhibitor that the assay system could indicate, without the need to dilute and re-assay the supernatant.

The Lineweaver-Burke plot indicated similar values for V_{max} , but different K_m values which indicated that both

DNJ and NOJ were competitive inhibitors. All the previous work examining the type of inhibition of α and β glucosidases by DNJ and NOJ showed the reaction to be competitive. For example, Reese et al. (1971) worked on the inhibition of α - and β - glucosidases by nojirimycin and found NOJ to inhibit competitively. However, not all the members of the nitrogen-containing alkaloids are competitive inhibitors of glucosidases. Saul et al., (1983) investigated the inhibition of castanospermine against β -glucosidase. Inhibition was found to be of a mixed type, because both the K_m and V_{max} were altered by increasing amount of inhibitor. The reaction was not competitive, but the binding of castanospermine was reversible, since the effect of the inhibitor could be diluted out.

The ratio of the K_i to the K_m was further indication of the effectiveness of the inhibitors. The ratio showed an almost ten-fold difference indicating both antibiotics were effective inhibitors of α -glucosidase. The similar inhibition caused by these inhibitors was a reflection of steric similarity between the inhibitor and substrate. There were fewer examples in the literature where DNJ and NOJ had been examined together. Schmidt et al. (1979) examined the effect of both inhibitors on sucrase, maltase, isomaltase and glucoamylase. The two inhibitors were equally effective against maltase and glucoamylase, whilst DNJ was ten fold more effective at inhibiting sucrase and isomaltase. They do not explain these differences beyond stating that, the inhibitory action by

nojirimycin has specific structural requirements.

The work in this project shows that similar concentrations of NOJ can cause equal amounts of inhibition as DNJ.

5.6.2 Antibiotic production titres

The DNJ basal medium was formulated by Ezure *et al.*, (1985). Analysis of fermentation of *S.lavendulae* strain MB-733 showed temporal differences in production of NOJ and DNJ. NOJ was produced first and reached a peak of production after only 48 hours. DNJ was not detected at this stage and did not reach its maximal value until 96 hours. The titres for NOJ were in decline at this point. The α -glucosidase assay could not differentiate between inhibition caused by either of these substances. Since the primary objective of the screen was to detect a DNJ- (or NOJ-) producing clone, no further attempt was made to separate the inhibition caused by each of the inhibitors. The data reflected a total inhibition and hence a total antibiotic titre of the two substances. However, even with this crude measure it can be seen that, *S.lavendulae* produced some inhibitor after only 48 hours and that total titres reached 3.5×10^{-5} M. in DNJ basal medium. These titres are in accord with those shown by Trew, (1992), using the trehalase assay system.

The titres recorded by *S.subbrutilus* in the DNJ medium also have some features in common with the profiles of production from Trew, (1992). Trew found that contrary

to *S.lavendulae*, there was no early production of DNJ by *S.subbrutilus*. When production did occur it was much later (after 100 hours) and both antibiotics were found to be produced together. Similar late production of inhibitors (after 6 days) was detected by the α -glucosidase assay in both media with this strain. However, the α -glucosidase assay did not allow differentiation of which antibiotics were produced at this stage. In addition, the overall titre for *S.subbrutilus* was much higher in previous work. Titres of 2.5×10^{-5} M. (comparable to levels with *S.lavendulae*) were recorded, which were ten times greater than titres recorded by the α -glucosidase assay. The discrepancy was difficult to explain, but the lower and later production of inhibitors by *S.subbrutilus* was a consistent finding throughout this project.

5.6.3 The use of statistical analysis to detect novel isolates detected by the screening data.

The detailed analysis of the screening procedure was an original investigation. Gene libraries are frequently screened using a bioassay, resistance against an antibiotic, or actual production of a pigment. This produces an immediate presence/absence result for any given transformant as to the possession of the relevant cloned DNA. The screening of enzyme activity looking for inhibition produced continuous, rather than discontinuous data. This required a more careful interpretation based

on comparisons with controls and differences between distributions and variances of populations. Both the transformed *S.lividans* and the untransformed *S.lividans* failed to produce any inhibitor. It was possible that failure to cause inhibition could be due to poor growth. However, this was unlikely since growth was clearly observed in the tested microtitre wells.

In the primary screen eight percent of the sample caused more than 50% inhibition. Additionally, there was a change in the distribution of the data which was attributed to a physiological response to transformation with a recombinant plasmid. Analysis of the primary screen data using Poisson distributions highlighted eight percent of transformants that produced significant inhibition. The 20% of the transformants of the primary screen that caused the most inhibition were re-screened. The Principal Components Analysis (PCA) identified the *S.lavendulae* population as distinct from the other populations. The separation of the data comprising the secondary screen from the other populations is intriguing and posed several questions, since the data from the secondary screen did not contain unusual statistical characteristics in terms of its mean or variation.

5.7 Conclusion

Transformants containing inserts of recombinant DNA caused an increase in inhibition of the assay compared to inhibition caused by transformants containing uncleaved vector. Some of these plasmids contained DNA insert sizes up to 10kb, but 75% of the plasmids were deleted. The inhibition detected in the primary screen was not maintained and there are two possible explanations for this. Firstly, it was possible that subculturing the transformants between primary, secondary and tertiary screening could have meant a loss of activity through plasmid deletion. Loss of the whole plasmid was unlikely since the sub culturing medium contained thiostrepton to maintain the plasmid. However, smaller scale deletions in non essential regions of cloned DNA were possible. The evidence from the plasmid digests does not support this explanation since plasmids were found to be of similar size after the repeated rounds of sub-culturing.

Secondly, the data in the primary screen could be a result of random variation in the 1380 transformants assayed. In the secondary and tertiary screens, when the number of transformants fell the number of individuals randomly producing a high percentage inhibition declined in accord. This assertion is further supported by evidence from the examination of the DNA content of eight recombinants from the tertiary screen. DNA inserts large enough to contain the DNJ gene cluster were not detected, although some clones may have produced some inhibitory products.

CHAPTER SIX

DISCUSSION

One of the original aims of the project was to investigate DNJ production by selected members of the *S.lavendulae* species group. The strain specific, rather than species specific nature of antibiotic production was highlighted in Chapter 3. Although this phenomenon was not new, the data provided an example of producers and non-producers of DNJ from members of the same taxonomic cluster group within the *Streptomyces*. Morphological similarity between the strains was no indication of the ability to produce DNJ.

S.lavendulae was also shown to produce the highest amounts of DNJ titre and liquid medium was identified as the optimal condition for high yields rather than a solid medium. The assay system used liquid DNJ basal medium which allowed optimal production of DNJ.

In conjunction with the shotgun cloning procedure mutants were characterised as an alternative method to achieve the cloning of the genes. One of the blocked mutants could synthesise DNJ when fed with NOJ, indicating the block was not in the final conversion step (NOJ to DNJ).

S.lividans is a genetically well characterised recipient for DNA with few restriction problems and pIJ702 is a frequently used high copy number vector. The resultant DNA insert sizes were small when transformants

were digested with *Bcl*I. A sample of the 2500 transformants indicated that only 5% (between 100 and 150) contained insert sizes greater than 10kb, the minimum estimated size of the biosynthetic gene cluster. Full consideration was taken of the possibility of using these transformants to complement DNJ blocked mutants. It was possible to protoplast one of the blocked mutants in tryptone soya broth (TSB), but regeneration of these on R5 or R2 at reproducibly high frequencies was difficult. However, regeneration of *S.lividans* on R2 has been accomplished (Nakano et al., 1981) and complementation of the blocked mutants provides the best opportunity for success in future work. Successful complementation of a mutant would allow the isolation of a gene, or part of the biosynthetic pathway, for NOJ production.

The use of large low copy number plasmids (pIJ916 and pIJ940) was an alternative strategy to achieve transformation of larger DNA inserts into *S.lividans*. Malpartida and Hopwood (1984) had failed to clone actinorhodin using pIJ702, but were successful when the low copy number SCP2 derivative pIJ922 was used. The use of low copy number plasmids means that any cloned DNA which results in the translation of toxic metabolites would be present at low copy number. The cloned DNA should therefore be less harmful inside the *S.lividans* cell. Chapter 4 provides ample discussion as to the nature of the large plasmid deletions that resulted from this cloning strategy. This strategy was thought

preferable to the use of cosmids which have been used by some workers. For example, the erythromycin O-transferase gene was transformed on a 4.5kb fragment of DNA into *E.coli*. To achieve the expression of the gene the DNA fragment was fused to a Lac Z promoter (Paulus et al., 1990). Kakinuma et al. (1991) used cosmids in a slightly different approach. Genomic libraries were made in *E.coli* which were probed with actinorhodin biosynthesis genes. This strategy did not require the expression of the genes in *E.coli*. Cosmids were generally used to clone large biosynthetic gene clusters. However, the DNJ pathway was not estimated to be more than 12kb. The relative small size of the gene cluster does not initially warrant the use of cosmids but their ability to package large fragments of DNA and exclude smaller fragments may be useful for future work.

The cause of the deletions could have been due to a restriction-modification system, which was checked using the KC301 phage. The analysis of the phage titres indicated that there was no restriction when cloning into *S.lividans*. There was evidence of a restriction-modification system in *S.lavendulae* which may affect plasmid stability and reduce the transformation frequency during self-cloning procedures. Self cloning may also result in problems of homologous recombination.

The use of the ZX1 mutant of *S.lividans* had two primary objectives. It was a *rec⁻* mutant which will prevent homologous recombination and was a modification mutant preventing restriction of *S.lividans* DNA in buffer

contaminated with ferrous iron. The ZX1 mutant caused no reduction in transformation frequency when pIJ702, pIJ916 and pIJ940 were used to generate transformants. The ZX1 mutant was also cited as effective at reducing intra-plasmid homologous and non-homologous recombination (Kieser and Hopwood, 1991). However, use of the ZX1 mutant was not always beneficial. There was no reduction in homologous recombination during either, conjugative crosses, protoplast fusion, or cloning systems involving low copy number plasmids (Kieser and Hopwood, 1991). The deleted plasmids could have been produced by restriction of *S.lividans* DNA in buffer containing ferrous iron. However, not all the plasmids, on any particular gel were subject to restriction. Contaminated buffer would surely affect all plasmids on any one gel. Additionally the gels were reproducible with fresh buffer, which would prevent the uncontrolled restriction of *S.lividans* DNA. The cause of these deletions was the key problem which hindered cloning of the biosynthetic gene cluster. Both cloning strategies (high and low copy number plasmids) produced transformants but only a minority of these had enough inserted DNA to contain the gene cluster. Therefore, either the number required for screening was large, rendering screening impractical or only a proportion of the genome of *S.lavendulae* or *S.subbrutilus* was examined.

The aims and objectives of the assay system have been achieved. The assay is not only capable of measuring titres of NOJ and DNJ but large numbers of clones

producing either of these antibiotics could be screened. The enzyme kinetics data provided supplementary information about the difference in inhibition between DNJ and NOJ. This information allowed the estimation of antibiotic titres in shake-flasks or microtitre plates using different media. Prior to any screening an assessment was made of the DNJ production in microtitre wells by *S.lavendulae*. These colonies were screened (positive control), as were colonies of *S.lividans* and *S.lividans* transformed with an uncleaved vector (negative controls).

There was a reduction in inhibition between the primary and secondary screens when transformants that contained high copy number plasmids were screened. *BclI*, *EcoRV* and *BglII* digests revealed the transformants only contained small inserts of DNA.

A similar pattern was observed when the low copy number plasmids were screened. Statistical analysis involving the mean, standard deviation and analysis of Poisson distributions was utilised. However, the initial level of inhibition detected in the primary screen was not maintained. Despite the data containing some elements of variation (shown by PCA analysis), almost all the interesting variation and inhibition was lost. There are accounts of proteases disabling enzymes and causing apparent inhibition, but this was unlikely in this assay, as no protease inhibition was detected in the untransformed or transformed *S.lividans*. Transformants digested with *BclI* were found to contain similar size DNA

inserts as transformants prior to screening. This indicated that plasmid deletion during the course of screening did not occur.

The activity detected in the screens was probably a result of physiological difference between transformants, due to position on the microtitre plate and inoculum size. The cloned DNA within the transformants, which may have caused inhibition in the primary screen, may have been lost due to sub-culturing between screening. Although it is possible to sub-culture transformants and assay for deletions, it would be expected that transformants contain more deletions after repeated sub-culturing. Analysis of *Bcl* I digests after the tertiary screen indicated this was not the case.

The cloned DNA accounted for increased inhibition in the primary screen, which was not present in either of the controls (*S.lividans* and transformed *S.lividans*). There were two main reasons to account for this: firstly it could have been due to the cloning of some inhibitory products on some of the larger DNA inserts. However, there was no evidence of DNA inserts being lost through sub culturing, therefore the inhibition should have been maintained through to the secondary and tertiary screen. Since the inhibition was lost, the second explanation of random variation in the population was more likely. Another possible explanation for the deletions was mutation through protoplasting. Hopwood (1981) indicated this could account for a high proportion of the deletions. The evidence indicated that the cloning of

DNA between *S.lavendulae* or *S.subtrutilus* into *S.lividans* was so inherently difficult that large inserts of DNA were rarely cloned. Large DNA inserts were either lethal at the transformation stage, or became deleted and only those plasmids retaining the replication functions and thiostrepton resistance survived. Transformants with these characteristics were repeatedly highlighted by the hybridisation experiment.

The potential benefits of cloning the DNJ biosynthetic gene cluster have not diminished since the start of the project. The application for glycosidase inhibitors has in fact diversified. The optimal future strategy to pursue the goal of cloning the genes is further use of blocked mutants and making a cosmid gene library. A greater variety of mutants would allow co-synthesis experiments and an assessment of the number of complementation groups. This should indicate the number of genes in the pathway, which should correlate with known information from the chemical pathway. Feeding of biochemical intermediates, for example fructose or an aminated form of fructose, will also identify the products and enzymes involved in the pathway. The pursuit of self-cloning is probably the optimal strategy as this does not require transformation of large DNA inserts. A small DNA insert to complement a mutation is all that is required. Cloning the genes would yield a better understanding of the control of antibiotic expression, identify the enzymes involved in biosynthesis and allow their exploitation in biotransformation.

REFERENCES

- Aharonowitz, Y. and A.L. Demain. 1977. Influence of inorganic phosphate and organic buffers on cephalosporin production by *Streptomyces clavuligerus*. Archives of Microbiology, 115: 169-173.
- Antonov, P.P., I .Ivanov and G.G. Markov. 1977. Heterogeneity of *Streptomyces* DNA. FEMS Letters 79: 151-154.
- Anzeveno, P.B. and L.J. Creemer. 1990. Efficient synthesis of (+)-nojirimycin and (+)-1-deoxynojirimycin. Tetrahedron Letters. 31: 2085-2088.
- Aoyagi, T., T. Yamamoto, K. Kojiri, F. Kojima, M. Hamanda, T. Takeuchi and H. Umezawa. 1978. Forphenicine, an inhibitor of alkaline phosphatase produced by actinomycetes. Journal of Antibiotics. 31: 244-246.
- Arai, T., S. Kuroda and Y. Mikami. Actinomycetes: The boundry Microorganisms. Toppan Co Ltd, Tokyo. 1-651.
- Arima, K., H. Imanaka, M. Kousaka, A. Fukada, and G. Tamura. 1965. Studies on Pyrrolinitrin, a new Antibiotic. I. Isolation and Properties of pyrrolinitrin. Journal of Antibiotics. 18: 201-204.
- Atsumi, A., K. Umezawa, H. Iinuma, H. Naganawa, H. Nakamura, Y. Iitaka and T., Takeuchi. 1990. Production, isolation and structure determination of a novel β -glucosidase inhibitor, cyclophellitol, from *phellinus* sp. The Journal of Antibiotics. 43: 49-53.
- Baltz, R.H. and J. Stonesifer. 1985. Phenotypic changes associated with the loss of expression of tylosin biosynthesis and resistance genes in *S.fradiae*. Journal Of Antibiotics. 38: 1226-1236.
- Bahal, V., Z. Hostalek and Z. Vanek. 1979. Anhydrotetracycline oxygenase activity and biosynthesis of tetracyclines in *Streptomyces aureofaciens*. Biotechnology Letters. 1: 177-182.

Behrmann, I., D. Hillman, A. Puhler, E. Strauch and W. Wohlleben. 1990. Overexpression of a *Streptomyces viridochromogenes* gene (gln II) encoding a glutamine synthase similar to those of eukaryotes confers resistance against the antibiotic phosphothricyl-alanyl-alanine. *Journal of Bacteriology*. 172: 5326-5334.

Bell, J.M., C. Falconer, J. Colby and E. Williams. 1987. Carbon monoxide metabolism by a thermophilic actinomycete, *Streptomyces* strain G26. *The Journal of General Microbiology*. 133: 3445-3456.

Bergey (1989) *Bergey's Manual of Systematic Bacteriology*. Vol. 4 Eds. Williams S., E. Sharp and J. Holt. Press: Williams and Wilkins.

Bibb, M.J., J.M. Ward and D.A. Hopwood. (1978). Transformation of plasmid DNA into *Streptomyces* protoplasts at high frequency. *Nature* 274: 398-400.

Bibb, M.J., J.M. Ward, T. Kieser, S.N. Cohen and D.A. Hopwood. 1981. Excision of chromosomal DNA sequences from *S.coelicolor* forms a novel family of plasmids detectable in *S.lividans*. *Molecular and General Genetics* 184: 230-240.

Biermann, M., R. Logan, K. O'Brian, E.T. Seno, R. Nagaraja Rao and B.E. Schoner. 1992. Plasmid cloning vectors and the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene*. 116: 43-49.

Bollen, M., A. Vandebroeck, and W. Stalmans. 1988. 1-deoxynojirimycin and related compounds inhibit glycogenolysis in the liver without affecting the concentration of phosphorylase a. *Biochemical Pharmacology*. 37: 905-909.

Booth, C. 1971. *Fungal Culture Media*. *Methods In Microbiology*. Eds. Booth, C. Academic Press, London and New York pp 49-94.

Brana, A.F., M.B. Manzanal, and C. Hardisson. 1980 Occurrence of polysaccharide granules in sporulating hyphae of *Streptomyces viridochromogenes*. *The Journal of Bacteriology*. 144: 1139-1142.

Brana, A.F., M.B. Manzanal and C. Hardisson. 1982. Characterisation of intracellular polysaccharides of *Streptomyces*. Canadian Journal of Microbiology. 28: 1320-1323.

Brown, A.G., B. Butterworth, M. Cole, G. Hanscomb, J.D. Hood, C. Reading and G.N. Rolinson. 1976. Naturally-occurring β -lactamase inhibitors with antibacterial activity. Journal of Antibiotics. 29: 668-669.

Bushall, M. 1988. Microbial Products: New Approaches. Eds. Baumberg, S., I. Hunter, and M. Rhodes. Cambridge University Press pp 95-120.

Cappelletti, L.M. and R. Spagnoli. 1983. Biological transformations of kanamycin A to amikacin (BBK-8). Journal of Antibiotics. 36: 927-930.

Chater, K.F. and C.J. Bruton. 1983. Mutational cloning in *Streptomyces* and the isolation of antibiotic production genes. Gene. 26: 67-78.

Chater, K. F. and C.J. Bruton. 1985. Resistance, regulatory and production genes for the antibiotic methylenomycin are clustered. The EMBO Journal. 1893-1892.

Chatterjee, S. and L.C. Vining 1981. Catabolite repression in *Streptomyces venezuelae*. Introduction of β -galactosidase, chloramphenicol production, and intercellular cyclic adenosine 3',5'-monophosphate concentrations. Canadian Journal of Microbiology. 28: 311-317.

Chida, N., Y. Furuno, and S. Ogawa. 1989. New synthesis of (+) and (-) nojirimycin from myo-inositol. Journal of Chemical Society Chemistry Communications. 1230-1231.

Cockrane, V.W., 1961. Physiology of actinomycetes. Annual Review of Microbiology. 15: 1-26.

Colegate, S.M., P.R. Dorling and C.R. Huxtable. 1979. A spectroscopic investigation of Swainsonine: an -mannosidase inhibitor isolated from *Swainsona canescens*. Australian Journal of Chemistry. 32: 2257-2264.

Crawford, D.L. 1988. Biodegradation of Agricultural and Urban Wastes. In. Actinomycetes In Biotechnology. Eds. Goodfellow, M., S.T. Williams and M. Mordarski. Academic Press San Diego. 433-459.

Crawford, D.L. and Sutherland. 1979. The role of actinomycetes in the decomposition of lignocellulose. Developements in Industrial Microbiology. 20: 143-151.

Dax, K., B. Gaigg, V. Grassberger, B. Kolblinger, and A.E. Stutz. 1990. Einfache synthesen von 1,5-didesoxy-1,5-imino-d-glucit (1-deoxynojirimycin) und 1,6-didesoxy-1,6-imino-d-glucit aus d-glucofuranurono-6,3-lacton. Journal of Carbohydrate Chemistry. 479-499.

Demain, A.L. 1981. Production of New Antibiotics by Directed Biosynthesis and by the use of Mutants. The Future of Antibiotherapy and Antibiotic Research. Eds: Ninet, L., Bost, E., Bouanchaud, D.H. and Florent J. London Academic Press 417-435.

Demain, A.L. 1986. Control of secondary metabolism in Actinomycetes. Biological, Biochemical and Biomedical Aspects of Actinomycetes. Ed. Szabo. G., S. Biro and M. Goodfellow. Proceedings of the Sixth International Symposium on Actinomycetes Biology, Debrecen, Hungary, 215-225. Akademiai Kiado, Budapest.

Deobald, L.A. and Crawford, D.L. 1987. Activities of cellulase and other extracellular enzymes during lignin solubilization by *S.viridosporus*. Applied Microbiology and Biotechnology. 26: 158-163.

Dussoix, D. and Arber W. 1962. Host specificity of DNA produced by *E.coli* II. Control over acceptance of DNA from infecting phage lambda. Journal of Molecular Biology. 5: 37-39.

Ebben, M.H., D.M. Spencer. 1978. The use of antagonistic organisms for the control of black root rot of cucumber. *Phomopsis sclerotioides*. Annals of Applied Biology. 89: 103-106.

Ensign, J.C. 1978. Formation, properties, and germination of actinomycete spores. Annual Review of Microbiology. 32: 185-219.

Evens, S.V., L.E. Fellows, T.K.M. Shing and G.W.J. Fleet. 1985. Glycosidase inhibition by plant alkaloids which are structural analogues of monosaccharides. Phytochemistry. 24: 1953-1955.

Everse J., C.L. Ginsburg and N.O. Kaplan. 1979. Immobilised enzymes in biochemical analysis. Methods in Biochemical Analysis. 25: 135-201.

Ezure Y., S. Maruo, K. Miyazaki and M. Kawamata 1985. Moranoline (1-Deoxynojirimycin) fermentation and its improvement. Agriculture and Biological Chemistry. 49: 1119-1125.

Fayerman, J.T. 1986. New developments in gene cloning in antibiotic producing microorganisms. Biotechnology. 4: 786-791.

Feitelson, J.S. and D.A. Hopwood. 1983. Cloning of a *Streptomyces* gene for an O-methyltransferase involved in antibiotic biosynthesis. *Molecular General Genetics*, 190: 394-399.

Feitelson, J.S., F. Malpartida and D.A. Hopwood. 1985. Genetic and biochemical characterisation of the red gene cluster of *Streptomyces coelicolor* A3(2). *The Journal Of General Microbiology*. 131: 2431-2441.

Fellows, L.E. 1986. The biological activity of polyhydroxyalkaloids from plants. *Pesticide Science* 17: 602-606.

Fisher, S.H., 1989. Glutamate synthesis in *Streptomyces coelicolor*. *Journal of Bacteriology*. 171: 2372-2377.

Flowers, T.H. and Williams S.T. 1977 Measurement of growth rates of *Streptomyces*: comparison of turbidimetric and gravimetric techniques. *Journal of General Microbiology*. 98: 285-289.

Fox, G.E., E. Stackebrandt, R.B. Hespell, J. Gibson, J. Maniloff, I. Dyer, R.S. Wolfe, W. Balch, R. Tanner, L. Magnum, L.B. Zablen, R. Blackmore, R. Gupta, K.R. Luehrsen, L. Bonen, B.J. Lewis, K.N. Chen and C.R. Woese 1980. The phylogeny of prokaryotes. *Science* 209: 457-463.

Fox, E.G. and E. Stackebrandt. 1987. The application of 16S rRNA cataloguing and 5S rRNA sequencing in bacterial systematics. *Methods In Microbiology*. 19: 405-438.

Furumai, T., K., Tekeda and M. Okanishi. 1982. Function of plasmids in the production of aureothricin. I. Elimination of plasmids and alteration of phenotypes caused by protoplast regeneration in *S.kasugaensis*. *Journal of Antibiotics*. 35:1367-1373.

Gaudreau, L.R., D. Boucher, J.M. Lavoie, R. Brzezinski, and C.V. Dery. 1992. Use of phage JHJ-1 to assess restriction in the genus *Saccharopolyspora*. *Canadian Journal Of Microbiology*. 38: 192-195.

Gersch, D., A. Skurk, and W. Romer. 1979. Phosphate inhibition of secondary metabolism in *Streptomyces hygroscopicus* and its reversal by Cyclic AMP. *Archives Of Microbiology*. 121: 91-96.

Gil, J.A., G. Naharro, J.R. Villanueva and J.F. Martin. 1981. Regulation by aromatic amino acids at the p-aminobenzoic acid synthetase level in *Streptomyces griseus*. Eds. Vazina C. and K Singh. Advances in Biotechnology. 3: 141-146.

Glaser, R. and A. Perlin. 1988. ¹H- and ¹³C.N.M.R. Studies on N-methyl-1-deoxynojirimycin, an α-D-glucosidase inhibitor. Carbohydrate Research. 182: 169-177.

Goodfellow, M., S.T. Williams, and G. Alderson. 1986a. Transfer of *Elytrosporangium brasiliense* Falco de Moraes et al., *Elytrosporangium carpinense* Falco de Moraes et al., *Elytrosporangium spirale* Falco de Moraes, *Microellobosporia cineria* Cross et al., *Microellobosporia grisea* (Konev et al.,) Pridam and *Microellobosporia violacea* (Tsyganov) Pridam to the genus *Streptomyces* with emended descriptions of the species. Systematic Applied Microbiology 8: 48-54.

Goodfellow, M., S.T. Williams and G. Alderson. 1986b. Transfer of *Chainia* species to the genus *Streptomyces* with amended description of species. Systematic Applied Microbiology 8: 55-60.

Goodfellow, M., S.T. Williams and G. Alderson. 1986c. Transfer of *Actinosporangium violaceum* Krasil'nikov and Yuan, *Actinosporangium vitaminophilum* Shomura et al. and *Actinopycnidium caeruleum* Krasil'nikov to the genus *Streptomyces*, with emended descriptions of the species. Systematic Applied Microbiology 8: 61-64.

Goodfellow, M., S.T. Williams and G. Alderson. 1986d. Transfer of *Kitosatoa purpurea* Matsumae and Hata to the genus *Streptomyces* as *Streptomyces purpureus*. Sytematic Applied Microbiology 8: 65-66.

Goodfellow, M., and E. Williams 1986. New strategies for the selective isolation of industrially important bacteria. Biotechnology and Genetic Engineering Review. 4: 213-262.

Gottlieb D. and E.B. Shirling. 1967. Co-operative description of type cultures of *Streptomyces*. The International *Streptomyces* Project. International Journal of Systematic Bacteriology. 17: 313-322.

Gruters, R.A., J.J. Neefjes, M. Tersmette, R.E.Y. Goede, A. Tulp, H.G. Huisman, F. Miedema, and H.L. Ploegh, 1987. Interference with HIV-induced syncytium formation and viral infectivity by inhibitors of trimming glucosidase. *Nature*. 330: 74-77.

Gullo, V.P, S.B. Zimmerman, R.S. Dewey, O. Hensens, and Hara, O., T. Murakami, I. Satoshi, H. Anzai, R. Itoh, Y. Kumada, E. Takano, E. Satoh, A. Satoh, K. Nagaoka, and C. Thomson. 1991. The bialophos biosynthetic genes of *Streptomyces viridochromogenes*: cloning, heterospecific expression, and comparison with the genes of *Streptomyces hygroscopicus*. *Journal Of General Microbiology*. 137: 351-359.

Hanozet, G., H. Pircher, Vanni, P. Oesch, B. and Semenza, G. 1981. An Example of enzyme hysteresis the slow and tight interaction of some fully competitive inhibitors with small intestinal sucrase. *The Journal of Biological Chemistry*. 256: 3703-3711.

Hardick, D., Hutchinson, D. and Trew S. 1992. Glucose is a precursor of deoxynojirimycin and deoxymannonojirimycin in *Streptomyces subbrutilus*. *Chemistry Communications*. 48: 6285-6296.

Hey-Ferguson, A, M. Mitchell and A.D. Elbein. 1973. Trehalose metabolism in germinating spores of *Streptomyces hygroscopicus*. *Journal of Bacteriology*. 116: 1084-1085.

Hockenhull, D.J.D., K.H. Fantes, M. Herbert and R. Whithead. 1954. Glucose utilisation by *S.griseus*. *The Journal of General Microbiology*. 10: 353-370.

Hodgson, D.A. (1982) Glucose repression of carbon source uptake and metabolism in *Streptomyces coelicolor* A3(2) and its perturbation in mutants resistant to 2-deoxyglucose. *Journal Of General Microbiology*. 128: 2417-2430.

Hohenschutz, L.D., E.A. Bell, P.J. Jewess, D.P. Leeworthy, D.P. Pryce, R.J. Arnold, E. Elardy. 1981. Castanospermine, a 1,6,7,8-tetrahydroxyoctahydroindolizine alkaloid from seeds of *castanospermine australe*. 20: 811-814.

Hopwood, D.A. 1972. Genetic analysis in microorganisms. In *methods in microbiology*. Eds: Norris J.R. and D.W. Ribbons. Academic Press London. 29-158.

Hopwood, D.A., H.M. Wright. 1978. Bacterial protoplast fusion recombination in fused protoplasts of *Streptomyces coelicolor*. *Molecular and General Genetics*. 162: 307-317.

Hopwood, D.A. 1981. Genetic studies with bacterial protoplasts. Annual Review of Microbiology. 35: 237-272.

Hopwood, D.A., T. Kieser, D.J. Lydiate and M.J. Bibb. 1983. Plasmids, recombination and chromosome mapping in *S.lividans* 66. Journal of General Microbiology 129: 2257-2269.

Hopwood D.A., G. Hintermann, T. Kieser, and H.M. Wright. 1984. Integrated sequences in 3 *Streptomyces* form related autonomous plasmids after transfer to *S.lividans*. Plasmid 11, 1-16.

Hopwood, D.A., M.J. Bibb, K.F. Chater, T. Kieser, C.J. Bruton, H.M. Kieser, D.J. Lydiate, C.P. Smith, J.M. Ward, and H. Schrempf. 1985. Genetic Manipulation of *Streptomyces*: a Laboratory Manual. F. Crowe and Sons Ltd, Norwich.

Hopwood, D.A. 1988. Towards an understanding of gene switching in *Streptomyces*, the basis of sporulation and antibiotic production. Proceedings of the Royal Society of London. 235: 121-138.

Hostalek, Z., M. Tinterova, V. Jechova, M. Blumauerova, J. Suchy and Z. Vanek. 1969. Regulation of biosynthesis of secondary metabolites biosynthesis of chlorotetracycline and tricarboxylic acid cycle activity. Biotechnology and Bioengineering. 11: 539-548.

Hotta, K., A. Takahasi, N. Saito, Y. Okami and H. Umezawa. 1983a. Multiple resistance and aminoglycoside antibiotics. Journal of Antibiotics. 36: 1789-91.

Hotta, K., A. Takahasi, Y. Okami and H. Umezawa. 1983b. Relationship between antibiotic resistance and antibiotic productivity in actinomycetes which produce amino-glycoside antibiotics. Journal of Antibiotics. 36: 1748-1754.

Hohenschutz, L.D., E.A. Bell, P.J. Jewess, D.P. Leworthy, R.J. Pryce, E. Arnold and J. Chardy. 1981. Journal of Phytochemistry. 20: 811-814.

Inoue, S., T. Tsuruoka and T. Niida. 1966. The structure of nojirimycin a piperidinose sugar antibiotic. The Journal of Antibiotics. 19: 288-291.

Ikeda, H., E. Seno, C.J. Bruton and K.F. Chater. 1984. Genetic mapping, cloning and physiological aspects of the glucose kinase gene of *Streptomyces coelicolor*. *Molecular and General Genetics*. 196: 501-507.

Ishida, N., K. Kumagai T.Niida, K. Hamamoto and T. Shomura. 1967. Nojirimycin a new antibiotic I. *The Journal of Antibiotics*. 20: 62-65.

Izumi, S., K. Nakahara, T. Gotoa, S. Hashimoto, T. Kino, M. Okuhara, H. Aoki and H. Imonaka. 1983. Anti-tumour effects of novel immunoactive peptides, FK-156 and its synthetic derivatives. *Journal of Antibiotics*. 36: 566-574.

Kakinuma, S., Y. Takada, H. Ikeda, H. Tanaka, S. Omura and D. Hopwood. 1991. Cloning of large DNA fragments, which hybridise with actinorhodin biosynthesis genes, from kalafungin and nanomycin, methyl ester producers and identification of genes for kalafungin biosynthesis of the kalafungin producer. *The Journal of Antibiotics*. 44: 995-1005.

Katz, E., C.J. Thomson and D.A. Hopwood. 1983. The cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. *Journal of General Microbiology* 129: 2703-2714.

Kendal, K. and J. Cullum. 1984. Cloning and expression of an extracellular-agarase gene from *Streptomyces coelicolor* A3(2) in *Streptomyces lividans* 66. *Gene*. 29, 315-321.

Kendal, K., U. Ali-Dunkrah and J. Cullum. 1987. Cloning of the galactokinase gene (galK) from *Streptomyces coelicolor* A3(2). *Journal Of General Microbiology*. 133: 721-725.

Kieser, H.M. (1989). A Mutation of *Streptomyces Lividans* which prevents intraplasmid recombination has no effect on chromosomal recombination. *Molecular General Genetics*. 220: 60-64.

Kieser, T. and D.A. Hopwood. 1991. Genetic manipulation of *Streptomyces*: new integrating vectors and methods for gene replacement. In Press.

Kinast, G., J.H. Guenther, M. Schedel and T. Michael. 1982. A four stage synthesis of 1-deoxynojirimycin with a biotransformation as the central reaction step. *Angewandte Chemie*. 93: 799-800.

Kinast, G. and M. Schedel. 1981. *Angewandte Chemie*. 20: 805-809.

Kinashi, H., M. Shamaji and A. Sakai. 1987. Giant linear plasmids in *Streptomyces* which code for antibiotic biosynthesis genes. *Nature* 328, 454-456.

Klaus, S. M. Hartmann, H. Krugal, M. Roth, F. Walter, Y.I. Rautenstein and N. Solovyeva. 1981. Restriction of *Streptomyces* phage SH5 by key endonuclease Shy I from *Streptomyces hygoscopicus* 0477. *Molecular and General Genetics*. 184: 286-288.

Kodama, Y., T. Tsuruoka, T. Niwa and S. Inouye. 1985. Molecular structure and glycosidase inhibitory activity of nojirimycin bisulfite adduct. *The Journal of Antibiotics*. 38: 116-118.

Korn-Wendish F., H.J. Kutzner. 1992. The Family Streptomycetaceae. *The Prokaryotes*. Vol I. Eds. Starr, M.P., H. Stolp, H.G. Truper, A. Butius and H.G. Schlegel. Springer-Verlag, Berlin, Heidelberg and New York. 921-995.

Kruger, D.H. and T.A. Bickle. 1983. Bacteriophage survival: multiple mechanisms for avoiding the DNA restriction systems of their hosts. *Microbiology Review*. 47: 345-360.

Kundu, P.K. and Nandi, B. 1985. Control of Rhizoctonia disease of cauliflower by competitive inhibition of a pathogen using organic amendments in soil. *Plant and Soil*. 33: 357-362.

Lacey, J. 1978. Ecology Of Actinomyces In Fodders and Related Substrates. 1976. In. *Proceedings in the International Symposium on Nocardia and Streptomyces*. Eds. Mordarski, M., W. Kurylowicz and J. Jeljaszewicz. Publisher, Gustav Fischer Verlag, Stuttgart. 161-170.

Lane, P., N. Galwey, N. Alvey. 1987. *Genstat 5 An Introduction*. Eds Payne, R.W. Clarendon Press Oxford.

Langley, D., M.J. Dawson and J.C. Vickers. 1991. *Streptomyces* from acidic environments. Selective isolation of strains, development of screening conditions and analysis of a screening campaign. Unpublished.

Larson J. and C.L. Hershberger. 1986. The minimal replicon of a *Streptomyces* plasmid produces an ultra-high level of plasmid DNA. *Plasmid* 15: 199-209.

Lebrihi, A., P. Germain and G. Lefebvre. 1987. Phosphate repression of cephamycin C and clavulanic acid production by *S.clavuligerus*. *Applied Microbiology and Biotechnology*. 26: 130-135.

Lechevalier, H.A. and Lechevalier, M.P. 1970. Chemical composition as a criterion in the classification of aerobic actinomycetes. *Journal of systematic Bacteriology* 20: 435-443.

Lilley, G., A.E. Clark and G.C. Lawrence. 1981. Control of the production of cephamycin C and thienamycin by *S.cattleia* NRRL 8057. *Journal Of Chemical Technology and Biotechnology*. 1 :127-135.

Locci, R. 1976. Developmental micromorphology of actinomycetes. 249-260. In T. Arai. *Actinomycetes- the boundary microorganisms*. Toppan Co. Ltd., Tokyo.

Lydate, D.J., F.Malpartida and D.A. Hopwood. 1985. The *Streptomyces* plasmid SCP2: its functional analysis and development into useful cloning vectors. *Gene* 35: 223-235.

Madry, N., R. Springmeyer, and H. Pape. 1979. Regulation of tylosin synthesis in *Streptomyces*. Effects of glucose analogues and inorganic phosphate. *European Journal of applied Microbiology and Biotechnology*. 7: 365-370.

Malpartida, F. and D.A. Hopwood. 1984. Molecular cloning of the whole biosynthetic pathway of a *Streptomyces* antibiotic and its expression in a heterologous host. *Nature*. 309: 462-464.

Malpartida, F. and D.A. Hopwood. 1986. Physical and genetic characterisation of the gene cluster for the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2). *Molecular and General Genetics*. 205: 66-73.

Malpartida, F., J. Niemi, R. Navarrete and D.A. Hopwood. 1990. Cloning and expression in a heterologous host of the complete set of genes for biosynthesis of the *Streptomyces coelicolor* antibiotic undecylprodigiosin. *Gene*. 93: 91-99.

Mansour, F.A., and Shady, M.A. 1984 Factors influencing the activity and production of nitrate reductase by certain local isolates of *Streptomyces*. *Egyptian Journal Of Botany*. 27: 105-117.

Martin, J.F., Demain, A.L. 1976. Control of phosphate of candidicin production. Biochemical and Biophysical research Communications. 71: 1103-1109.

Martin, J.F. and L.E. McDaniel. 1975. Kinetics of biosynthesis of polyene macroglide antibiotics in batch cultures: cell maturation time. Biotechnology and Bioengineering XVII: 925-938.

Matsushima, P. and R.H. Baltz. 1985. Efficient plasmid transformation of *Streptomyces ambofaciens* and *Streptomyces fradiae* protoplasts. Journal of Bacteriology. 163: 180-184.

Mayfield, C.J., S.T. Williams, S.M. Ruddick and H.L. Hatfield. 1972. Studies on the ecology of actinomycetes in soil. Observations on the form and growth of *Streptomyces* in soil. Soil Biology and Biochemistry. 4: 79-91.

Mazodier, P., R. Petter and C. Thomson. 1989. Inter generic conjugation between *Escherichia coli* and *Streptomyces* species. Journal of Bacteriology. 171: 3583-3585.

McBride, M. and J. Ensign. 1987. Metabolism of endogenous trehalose by *Streptomyces griseus* Spores by spores or cells of other actinomycetes. Journal of Bacteriology. 169, 5002-5007.

McHenney, M. and R.H. Baltz. 1991. Transduction of plasmid DNA in *Streptomyces* spp and related genera by bacteriophage FP43. Journal of Bacteriology. 170: 2276-2282.

McNeil, D. 1988. Characterisation of a unique methyl-specific restriction system in *Streptomyces avermitilis*. Journal of Bacteriology. 170: 5607-5612.

Miyairi, N., T. Miyoshi, H. Aoki, M. Kohsaka, H. Ikushima, K. Kunugita, H. Sakai and H. Imanaka. 1970. Studies on Thiopeptin Antibiotics. I. Characteristics of Thiopeptin Antibiotics. Journal of Antibiotics 23: 113-119.

Montefiori, D.C., W.E. Robinson JR., and W. Mitchell. 1988. Role of protein N-glycosylation in pathogenesis of human immunodeficiency virus type 1. Proceedings of the National Academy of Sciences USA. Medical Sciences. 85: 9248-9252.

Mordarski, M., J. Wieczorek, B. Jaworska. (1970). On the condition of amylase production by actinomycetes. *Archivum Immunologiae et Therapiae Experimentalis*. 18: 375-381.

Murakami, T., H. Anzai, S. Imai, A. Satoh, K. Nagoaka and C.J. Thomson. 1986. The bialophos biosynthetic genes of *Streptomyces hygroscopicus*: molecular cloning and characterisation of the gene cluster. *Molecular and General Genetics*. 205: 42-50.

Murao, S. and S. Miyata. 1980. Isolation and characterisation of a new trehalase inhibitor, S-GI. *Agricultural and biological chemistry*. 44: 219-221.

Nakano, M.M., H. Ishihara and H. Ogawara. 1981. Fusion of protoplasts of *Streptomyces Lavendulae*. *The Journal of Antibiotics*. 35: 359-363.

Nakano, M.M., H. Mashiko and H. Ogawara. 1984. cloning of the kanamycin resistance gene from a kanamycin producing *Streptomyces* species. *Journal of Bacteriology*. 157: 79-83.

Neesen, K. and G. Volckaert. 1989. Construction and shuttling of novel bifunctional vectors for *Streptomyces* spp and *Escherichia coli*. *Journal of Bacteriology*. 171: 1569-1573.

Niwa, T., S. Inouye, T. Tsuruoka, Y. Koaze and T. Niida, 1970. "Nojirimycin" as a potent inhibitor of glucosidase. *Agricultural and Biological Chemistry*. 34: 966-968.

Ohnuki T., T. Imanaka, S. Aiba. 1985. Self-cloning in *Streptomyces griseus* of an str gene cluster for streptomycin biosynthesis and streptomycin resistance. *Journal of Bacteriology*. 164: 85-94.

Okamura, H., A. Koki, M. Sackamoto, K. Kubo, Y. Mutoh, Y. Fukagawa, K. Kouno, K. Shimauchi, Y. Ishikura and J. Lein. 1979. Microorganisms producing a new β -Lactam Antibiotic. *Journal of Fermentation Technology*. 57: 265-272.

Okanishi, M., K. Katagari, T. Fumumai, K. Takeda, K. Kawaguchi, M. Saith and S. Nabeshima. 1983. Basic techniques for DNA cloning and conditions required for *Streptomyces* as a host. *Journal of Antibiotics*. 36: 99-108.

Omoto, S., T. Shomura, H. Suzuki and S. Inouye. 1979. Studies on actinomycetales producing antibiotics only on agar culture. II. isolation, structure and biological properties of N-carbamoyl-D-glucosamine. *Journal of Antibiotics*. 32: 436-441.

Omura, S., N. Sadakane, Y. Tanaka and H. Matsubara. 1983. Chimeramycins: new macroglide antibiotics produced by hybrid biosynthesis. *Journal of Antibiotics*. 36: 927-930.

Palmer T. 1981. Understanding enzymes. 119-169. Ellis Horwood Ltd. Halsted Press.

Paulus, T.J., J.S. Tuan, V.E. Luebke, G.T. Maine, J.P. De Witt and L. Katz. 1990. Mutation and cloning of *eryG*, the structural gene for erythromycin O-methyltransferase from *Saccharopolyspora erythraea*, and expression of *eryG* in *Escherichia coli*. *Journal of Bacteriology*. 172: 2541-2545.

Pederson, R. L., M.J. Kim and C. Wong. 1988. A combined chemical and enzymatic procedure for the synthesis of 1-deoxynojirimycin and 1-deoxymannojirimycin. *Tetrahedron Letters*. 29: 4645-4648.

Pfennig, N. 1958. Beobachtungen des wachstumsverhaltens von streptomyceten auf rossicholodny-aufwuchsplatten im boden. *Archives Mikrobiol*. 31: 206-216.

Phillips, L., E.M.H. Wellington, S.B. Rees, L.S. Jun and G.P. King. 1992. The distribution Of DNA sequence hybridising with antibiotic production and resistance gene probes within type strains and wild isolates of *Streptomyces* species. *The Journal Of Antibiotics*. 45: 1481-1491.

Puls, W., U. Kelp., H. P. Krause, L. Muller, D.D. Schmidt, G. Thomas and E. Truscheit. 1980. Pharmacology of a glucosidase inhibitor. *Frontiers Of Hormone Research*. 7: 235-247.

Rao, R.N., M.A. Richardson and N. Kuhstoss. 1987. Cosmid shuttle vectors for cloning and analysis of *Streptomyces* DNA. *Methods In Enzymology*. 153: 166-194. Academic Press.

Randazzo, R., G. Sermonti, A. Carere and M. Bignami. 1973. Comutation in *Streptomyces*. *Journal of Bacteriology* 113: 500-501.

Reese, E.T., F.W. Parish and M. Ettlinger. 1971. Nojirimycin and D-glucono-1,5-lactone as inhibitors of carbohydrases. *Carbohydrate Research*. 18: 381-388.

Reitz, A. B. and E.W. Baxter. 1990. Pyrrolidine and piperidine aminosugars from dicarbonyl sugars in one step. Concise synthesis of 1-deoxynojirimycin. *Tetrahedron Letters*. 31: 6777-6780.

Ribbons, D.W. 1970. Quantitative relationships between growth media constraints and cellular yields and composition. *Methods in Microbiology*. Ed. Morris J.R. and D.W. Ribbons. Academic Press London and New York. pp 297-304.

Saul, R., J.P. Chambers, J.R. Molyneux, A.D. Elbein. 1983. Castanospermine, a tetrahydroxylated alkaloid that inhibits β -glucosidase and β -glucocerebrosidase. *Archives of Biochemical Biophysics*. 221: 593-597.

Schatz, A., E. Bugie and S.A. Waksman. 1944. Streptomycin, a substance exhibiting antibiotic activity against Gram-positive and Gram-negative bacteria. *Proceedings of the Society for Experimental Biology and Medicine*, 55: 66-69.

Schmidt, D.D., W. Frommer, L. Muller, E. Truscheit. 1979. Glucosidase-inhibition aus bazillen. *Naturwissenschaften* 66: 584-585.

Schueller, A.M. and F. Heiker. 1990. Synthesis of 2-acetamido-1,2,5-trideoxy-1,5-imino-D-galacto-nojirimycin (2-acetamido-1,2,5-trideoxy-1,5-imino-D-galactitol) from 1-deoxynojirimycin. *Carbohydrate research*. 203: 308-313.

Scofield, A.M., L.E. Fellows, J.R. Nash and G.W.J. Fleet. 1986. Inhibition of mammalian digestive disaccharidases by polyhydroxy alkaloids. *Life Science*. 39: 645-650.

Scott, B.R. and T. Alderson. 1971. The random (non-specific) forward mutational response of gene loci in *Aspergillus conidia* after photosensitization to near ultraviolet light (365nm) by 8-methoxysoralen. *Mutagen Research* 12: 29-34.

Shapiro, S. and L.C. Vining. 1983 Nitrogen metabolism and chloramphenicol production in *Streptomyces venezuelae*. *The Canadian Journal of Microbiology*. 29: 1706-1714.

Shapiro, S. and L.C. Vining. 1984. Suppression of nitrate utilisation and its relationship to chloramphenicol production in *Streptomyces venezuelae*. *Canadian Journal of Microbiology*. 33: 798-804.

Sharples, G.P., S.T. Williams and R.M. Bradshaw. 1974. Spore formation in the Actinoplanaceae (Actinomycetales) *Archives of Microbiology*. 101: 9-20.

Shimizu, H., H. Tsuchie, K. Yoshida, S. Morikawa, T. Tsuruoka, H. Yamamoto, H. Ushijima, and T. Kitamura. 1990. Inhibitory effect of novel 1-deoxynojirimycin derivatives on HIV-1 replication. *Aids*. 4: 975-979.

Shimizu, H., H. Tsuchie, H. Honma, K. Yoshida, T. Tsuruoka, H. Ushijima, and T. Kitamura. 1990. Effect of N-(3-phenyl-2-propenyl)-1-deoxynojirimycin on the lectin binding to HIV-1 glycoproteins. Japanese Journal of Medical Science and Biology. 43: 75-87.

Shomura, T., J. Yoshida, S. Amani, M. Kojima. S. Inouge and T. Niida. 1979. Studies on Actinomycetales producing antibiotics only on agar culture. Screening taxonomy and morphology-productivity relationship of *S.halstedii* strain SF-1993. Journal of Antibiotics. 32: 427-435.

Smith, D.J., M.K.R. Burnham, J.H. Bull, J.E. Hodgson, J.M. Ward, P. Browne, J. Brown, B. Barton, A.J. Earl and G. Turner. 1990. β -Lactam antibiotics biosynthetic genes have been conserved in clusters in prokaryotes and eukaryotes. The EMBO Journal. 9: 741-747.

Smith, C.P. and K. F. Chater. 1986. Physiology genetics and molecular biology of glycerol utilisation in *Streptomyces coelicolor*. Fifth International Symposium on the Genetics of Industrial Microorganisms. 7-14 Eds. Alacevic M., D. Hranueli and Z. Toman.

Smith, M.C.M., C.J. Ingham, C.E. Owen, and N.T. Wood, 1992. Gene expression in the *Streptomyces* temperate phage ϕ C31. Gene. 115: 43-48.

Sneh N. and Y. Henis. 1972. Production of anti-fungal substances active against *Rhizoctonia solani* in chitin-amended soil. Phytopathology. 62: 595-600.

Soliveri, J., A. Mendoza and M. Arias. 1988. The effect of different nutrients on the production of polyene antibiotics PA-5 and PA-7 by *Streptoverticillium* sp 43/16 in chemically defined media. Applied Microbiology and Biotechnology. 28: 254-257.

Solomons, G.L. 1969. Materials and Methods in Fermentation. Academic Press London and New York.

Stackebrandt, E. and C.R. Woese. 1981. Towards a phylogeny of the actinomycetes and related organisms. Current Microbiology. 32: 1-31.

Stackebrandt, E. and O. Charfreitag. 1989. Partial 16S rRNA primary structure of five *Actinomyces* species: phylogenetic implications and development of an *Actinomyces israelii* specific oligonucleotide probe. Journal of General Microbiology. 136: 37-43.

Stein, D.C. and S.N. Cohen. 1989. The cloned regulatory gene of *Streptomyces lividans* can suppress the pigment deficiency phenotype of different developmental mutants. Journal of Bacteriology. 171: 2258-2261.

Stone, M.J. and D.H. Williams. 1992. On the evolution of functional secondary metabolites (natural products). *Molecular Microbiology*. 6: 29-34.

Sugimoto Y., H. Suzuki, H. Yamaki, T. Nishimura and N. Tanaka. 1982. Mechanism of Action of 2-crotonyloxymethyl-4,5,6-trihydroxycyclohex-2-enone, a SH inhibitory anti-tumour antibiotic and its effect on drug-resistant neoplastic cells. *Journal of Antibiotics*. 35: 1222-1230.

Szwajcer, E., A. Szewczuk, M. Mordarski. 1981. Application of immobilised aminoacylase from *Micrococcus agilis* for isolation of D-phenylglycine from its racemic mixture. *Biotechnology and Bioengineering*. 23: 1675-1681.

Thomson, C.J. and H. Anzai. 1986. Organisation and regulation of the bialaphos biosynthetic genes. Fifth international symposium on the genetics of industrial microorganisms. Eds. Alecavic, M., D. Hranveli, and Z. Toman.

Thomson, C.J., J.M. Ward and D.A. Hopwood. 1982. Cloning of antibiotic resistance and nutritional genes in streptomycetes. *Journal of Bacteriology*. 151: 668-677.

Trew, S. (1992). Postgraduate Thesis. Studies on the biosynthesis of 1-deoxynojirimycin.

Tronick, S.R., J.E. Ciardi and E.R. Stadtman. 1973. Comparative biochemical and immunological studies of bacterial glutamine synthases. *Journal of Bacteriology*. 115: 858-868.

Truscheit, E., W. Frommer, B. Junge, M. Muller, D. D. Schmidt and W. Wingender. 1981. Chemistry and biochemistry of microbial α -glucosidase inhibitors. *Angewandte chemie*. 20, pp 744-761.

Tuan, J.S., M. Weber, M.J. Staver, J. O.Leung, S. Donadio and L. Katz. 1990. Cloning of Genes Involved in Erythromycin Biosynthesis from *Saccharopolyspora erythraea* using a Novel Actinomycete-*Escherichia coli* Cosmid. *Gene*. 90: 21-29.

Turpin, P.E., Dhir, V.K., Maycroft, K.A., Rowlands, C. and E.M.H. Wellington. 1992. The effect of *Streptomyces* species on the survival of *Salmonella* in soil. *FEMS Microbial Ecology* 101 271-280.

Umezawa H. 1972. Enzyme inhibitors of Microbial Origin. University of Tokyo Press, Tokio.

Umezawa H., Y. Aoyagi, H. Suda, M. Hamada and T. Takeuchi. 1976. Bestatin, an inhibitor of aminopeptidase B, produced by actinomycetes. *Journal of Antibiotics* 29: 97-99.

Umezawa, H. 1988. Low-molecular-weight enzyme inhibitors and immunomodifiers. In. *Actinomycetes In Biotechnology*. Academic Press, San Diego. 286-325.

Vandamme, E.J. and Voets, J.P. 1974. Microbial penicillin acyclases. *Advances In Applied Microbiology*. 17D: 311-369.

Vara, J.A., D. Pulido, R.A. Lacalle and A. Jimenez. 1988. Two genes in *Streptomyces alboniger* puromycin biosynthesis pathway are closely linked. *Gene*. 69: 135-140.

Vara, J., F. Malpartida, D.A. Hopwood. and A. Jimenez. 1985. A cloning and expression of a puromycin N-acetyl transferase from *Streptomyces alboniger* in *Streptomyces lividans* and *Escherichia coli*. *Gene* 33: 197-206.

Vilches, C., C. Mendez, C. Hardisson and J.Salas. 1990. Biosynthesis of Oleandomycin by *Streptomyces antibioticus*: influence of nutritional conditions and development of resistance. *Journal of General Microbiology*. 136: 1447-1454.

Wax, R., L. Snyder, and L. Kaplan. 1982. Introduction of glutamine synthase by ammonia shock in the Gram positive bacterium *S.cattleia*. *Applied and Environmental Microbiology*. 44: 1004-1006.

Wellington, E.M. and M. Al-Jawadi. 1985. Production of a polyene antifungal agent by members of the *Streptomyces lavendulae* species group. *Sixth International Symposium on Actinomycetes Biology*, 1985. Eds: Szabo, G., S. Biro and M. Goodfellow. pp 267-270.

Williams, S.T. 1978. *Streptomyces* in the soil ecosystem. In *Nocardia and Streptomyces*. Eds. Mordarski, M., W. Kurylowicz and J. Jeljaszewicz. Fisher Verlag. Stuttgart and New York.

Williams, S.T., M. Goodfellow, G. Alderson, E.M.H. Wellington, P.H.A. Sneath and M.J. Sackin. 1983. Numerical classification of *Streptomyces* and related genera. *Journal of General Microbiology*. 129: 1743-1813.

Williams, S.T. and C.I. Mayfield. 1971. Studies on the ecology of actinomycetes in soil. III. The behaviour of neutrophilic streptomycetes in acid soil. *Soil Biology Biochemistry*. 3: 197-208.

Williams, S.T., F.L. Davies, C.I. Mayfield and M.R. Khan. 1971. Studies on the ecology of actinomycetes II. The pH requirements of streptomycetes from two acid soils. *Soil and Biology Biochemistry*. 3: 187-195.

Williams, S.T. and C.S. Robinson. 1981. The role of streptomycetes in decomposition of chitin in acidic soils. *Journal of General Microbiology*. 127: 55-63.

Witt, D., E. Stackebrandt 1990. Unification of the genera *Streptoverticillium* and *Streptomyces* and amendment of *Streptomyces* Waksman and Henrici 1943, 339AL. *Systematic Applied Microbiology*. 13: 361-371.

Woodman, R., T. Miller, H. Floss, and W. Strohl. 1991. In press. pIJ101-like plasmids from *Streptomyces azureus*.

Xia, T. and R. Jiao (Chiao, J.S), 1980, Studies on glutamine synthase from *Streptomyces hygroscopicus* var *jinggangensis*. *Scientia Sin. (Ser.B)* 29: 379-388.

Yagi, M., T. Kuono, Y. Aoyagi and H.Murai. 1976. *Nippon Nogei Kagaku Kaishi*. 50: 571-572.

Yegneswaran, P.K., M.R. Gray and D.W.S. Westlake, 1988. Effects of reduced oxygen on growth and antibiotic production in *S.clavuligerus*. *Biotechnology Letters*. 10, 479-484.

Zanca, D. M. and J. F. Martin. 1983. Carbon catabolite repression of the conversion of penicillin N into cephalosporin C. *Journal of Antibiotics*. 36: 700-708.

Zeigler, T., A. Straub, and F.Effenberger. 1988. Enzyme-catalysed synthesis of 1-Deoxymannojirimycin, 1-Deoxynojirimycin and 1,4-Dideoxy-1, 4-imino-D-arabinitol. *Angewandte Chemie*. 100: 716-717.

Zhou, X., Z. Deng, J.L. Firmin, D.A. Hopwood and T. Kieser. 1988. Site-specific degradation of *Streptomyces lividans* DNA during electrophoresis in buffers contaminated with ferrous iron. *Nucleic Acids Research*. 16:4341-4352.

APPENDICES

APPENDIX 1

OATMEAL AGAR

Fine oats	20g
yeast extract	1g
Lab M Agar (No 1)	15g
Distilled water	1000ml

pH 7.0 sterilise 15min at 121°C.

MALIC ACID MEDIUM

Malic Acid	10g
Asparagine	1g
MgSO ₄ . 7H ₂ O	0.5g
K ₂ HSO ₄	1g
Trace Elements Solution (T.E.S.)	1ml
Distilled water	1000ml

Adjust to pH 7.0 sterilise 15mins at 121°C.

Trace Element Solution (TES)

FeSO₄ 7H₂O (0.1g), MnCl₂ (0.1g), ZnSO₄.7H₂O (0.1g) in 100ml. Sterilise for 15mins at 121°C.

MINIMAL GLUCOSE AGAR

Glucose	2g
Asparagine	1g
K ₂ HPO ₄	0.5g
MgSO ₄ . 7H ₂ O	0.5g
Lab M Agar (No 1)	15g

Trace element solution 1ml

Distilled water 1000ml

Adjust to pH 7.4 and sterilise for 15 mins at 121°C.

Trace Element Solution

FeSO₄.7H₂O (0.1g), CuSO₄.5H₂O (0.1g) ZnSO₄.7H₂O (0.1g) in 100ml. Sterilise for 15mins at 121°C.

INORGANIC SALTS AND STARCH AGAR (ISP4)

Soluble starch 10g

K₂HPO₄ 1g

MgSO₄. 7H₂O 1g

NaCl 1g

(NH₄)₂SO₄ 2g

CaCO₃ 2g

Lab M Agar 15g added where appropriate

Distilled water 1000ml

Sterilise for 15 mins at 121°C.

Aseptically add 1ml of Trace elements solution.

FeSO₄ 7H₂O (0.1g), MnCl₂ (0.1g), ZnSO₄.7H₂O (0.1g) in 100ml. Sterilise for 15mins at 121°C.

DNJ MEDIUM (Ezure et al., 1985)

Soluble starch 20g

Soyabean meal 10g

MgSO₄. 7H₂O 0.5g

KCl 0.5g

NaCl 5g

NaNO₃ 2g

CaCO₃ 3.5g

Lab M Agar 15g added where appropriate

Distilled water 1000ml

Adjust to pH 7.2 and sterilise for 15 mins at 121°C.

GLYCEROL AND PROLINE MEDIUM

Glycerol	14.75g
NH ₄ Cl	3.21g
K ₂ HPO ₄	2g
3-[N-Morpholino] propane sulfonic acid (MOPS Buffer)	15.7g
Lab M Agar (No 1)	15g

Sterilise and complete by aseptic addition of
1ml/l of 2M MgSO₄, 1ml/l of 0.2M CaCl₂ and
1ml of Trace element solution.

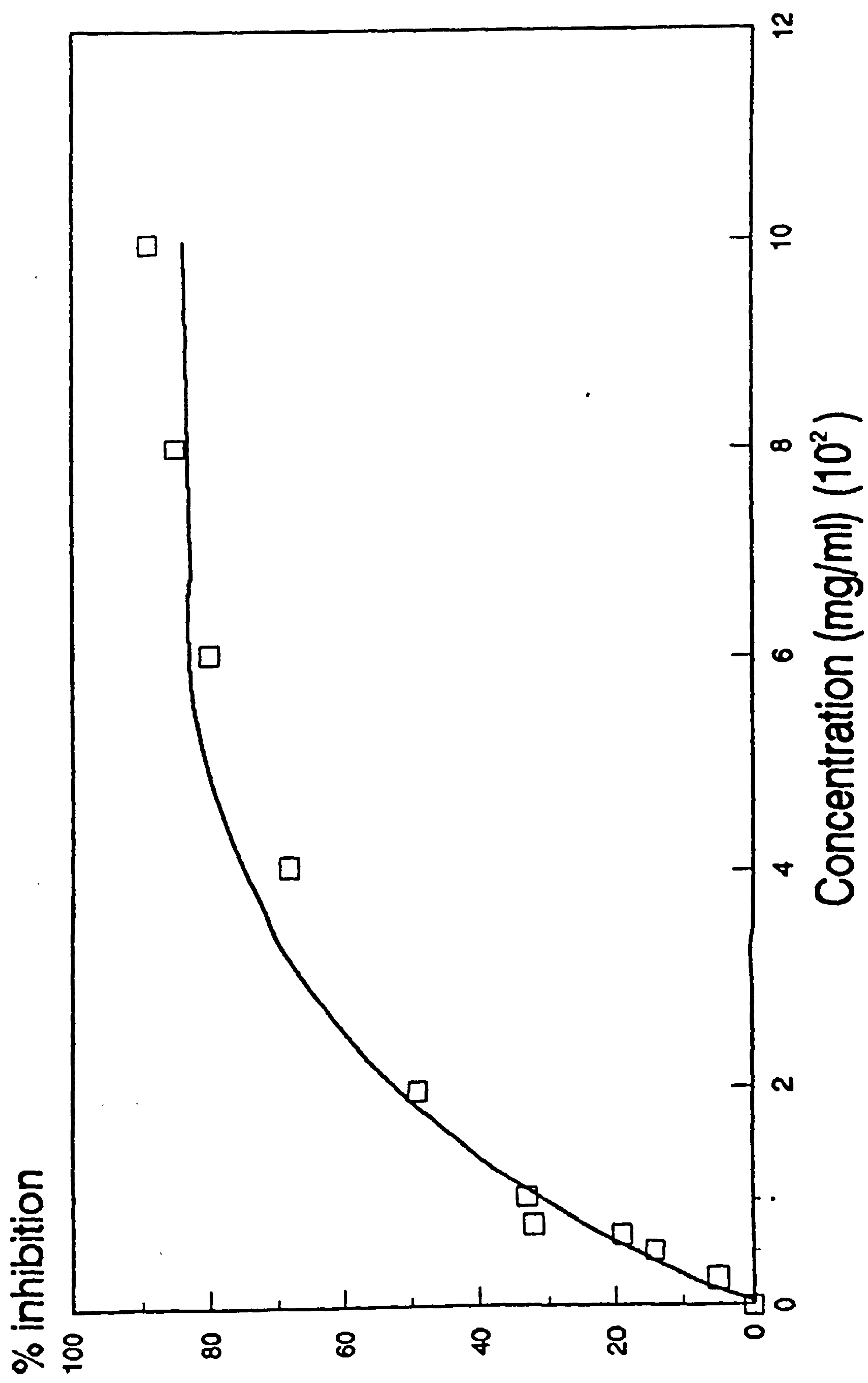
Trace element solution consists of the following
solutions 0.1M. ZnCl₂ 15ml, MnCl₂ 5mls, H₃BO₃ 5ml,
CuCl₂.5H₂O 2.5ml, Na₂MoO₄ 1ml, CoCl₂ 1ml, FeCl₃
32.5ml, NaI 2.5ml, and make up to 100ml with
distilled water.

STARCH AND GLYCEROL AGAR ISP5

Soluble starch	10g
Glycerol	10g
Asparagine	1g
K ₂ HPO ₄	1g
MgSO ₄ . 7H ₂ O	1g
NaCl	0.5g
Lab M Agar (No 1)	15g
Distilled water	1000ml

Sterilise 15 mins/15p.s.i. Aseptically add 1ml of
Trace element solution (as for malic acid medium).
Adjust to pH 6.8 if necessary.

Fig 7.1 **Inhibition of Trehalase by DNJ.**



ZX1 Medium

Soluble starch	10g
NaCl	1g
(NH ₄) ₂ SO ₄	2g
K ₂ HPO ₄	1g
CaCO ₃	2g
MgSO ₄ . 7H ₂ O	2g
Tryptone	2g
Inorganic salt solution	1ml/l
Lab M Agar 15g added where appropriate	
Distilled water	1000ml

Adjust to pH 7.1 if necessary

Sterilise for 15mins at 121°C and aseptically add 1ml of
Trace element solution (as for malic acid medium)

APPENDIX 2

R5

Sucrose	103g
K ₂ SO ₄	0.25g
MgCl ₂ .6H ₂ O	10.12g
Glucose	10g
Difco casaminoacids	0.1g
Difco yeast extract	5g
N-Tris-(hydroxymethyl)-methyl-2-amino -ethanesulphonic acid (TES Buffer)	5.73g
Difco Bacto-agar	22g
Distilled water	1000ml

Sterilise for 15 mins at 121°C and aseptically add the following. Adjust to pH 7.1 if necessary.

KH ₂ PO ₄	5ml
CaCl ₂ .6H ₂ O (5M)	2ml
L-proline (20%)	7.5ml
NaOH (1N)	3.5ml

YEAST EXTRACT-MALT EXTRACT MEDIUM (YEME)

Difco yeast extract	3g
Difco Bacto-peptone	5g
Oxoid malt extract	3g
Glucose	10g
Sucrose	340g

Sterilise for 15 mins at 121°C and aseptically add MgCl₂.6H₂O (2.5M) (2ml/l) and Glycine 25ml/l

ACID PHENOL/CHLOROFORM

Phenol	5g
Chloroform	5ml
Distilled water	1ml
8-hydroxyquinoline	5mg

NEUTRAL PHENOL/CHLOROFORM

Acid phenol/chloroform equilibrated with 0.5 vol 1M Tris pH 8.8 and then with 0.5 vol 0.1M Tris pH 8.0

TE BUFFER

10mM Tris-HCL pH 8.0

1mM EDTA pH 8.0.

Sterilise at 121°C for 15mins.

TRIS-BORATE BUFFER (TBE)

Tris base 54g

Boric acid 27.5g

EDTA 0.5M 20ml pH 8.0

PROTOPLAST BUFFER (Okanishi et al. 1974; Hopwood and Wright, 1978)

Make up the following basal solution

Sucrose 103g

K₂SO₄ 0.25g

MgCl₂ 6H₂O 2.02g

Trace element solution 2ml

Make up to 800ml with distilled water

Dispense to 80ml aliquots and sterilise for 15mins at 121°C. Before use, add to each flask in order aseptically and sterile solutions of:

K₂HPO₄ (0.5%) 1ml

CaCl₂·2H₂O (3.68%) 10ml

T.E.S. buffer (5.73%, adjusted to pH 7.2)

Trace element solution.

ZnCl ₂	40mg
FeCl ₂ 6H ₂ O	200mg
CuCl ₂ 2H ₂ O	10mg
MnCl ₂ 2H ₂ O	10mg
Na ₂ B ₄ O ₇ 10H ₂ O	10mg
(NH ₄) ₆ Mo ₇ O ₂₄ 4H ₂ O	10mg

LIGATION BUFFER (Hopwood et al., 1985)

Tris HCL pH 7.5	30mM
MgCl ₂ 6H ₂ O	6mM
Dithiothreitol	10mM
Bovine serum albumin	50μl/ml
ATP from 100mM stock solution	0.5mM
Normally added as ten times buffer.	

T (TRANSFORMATION) BUFFER (Thomson et al., 1982)

Sucrose (10.3%)	25ml
Distilled water	75ml
T.E.S.	0.2ml
K ₂ SO ₄	1ml

To 9.3ml of the above solution add:

CaCl ₂ 2H ₂ O (5M)	2ml
Tris-maleic acid buffer	0.5ml

For use, 3 parts by volume of the above solution was added to one part by weight of PEG 1000 previously sterilised at 121°C for 15mins.

HYBRIDISATION SOLUTION

Five times SSC. Made from dilution of 20 times SSC stock solution, (3M NaCl; 0.3M Na-citrate; pH 7.0 at 20°C). N-lauroylsarcosine, Na-salt (Sigma) 0.1% (w/v). SDS 0.02% (w/v), Blocking agent 1% (w/v).

BUFFER 1 (Tris-Hcl/NaCl buffer)

100mM Tris-Hcl; 150mM NaCl; pH 7.5 (20°C)

BUFFER 2 (Blocking buffer)

Blocking agent 0.5% (w/v) in buffer 1.

BUFFER 3 (Tris-Hcl/NaCl/MgCl₂ buffer)

100mM Tris-HCL; 100mM NaCl; 50mM MgCl₂; pH 9.5 (20°C)

COLOUR SOLUTION

45μl Nitroblue tetrazolium salt (in 70% v/v dimethylformamide).

35μl X-phosphate-solution

Buffer 3 10ml